

ETS
ISSUE BINDER

EXPOSURE
A REVIEW OF
THE LITERATURE

VOLUME II

2023381050

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exposure to tobacco during the past few days. Nicotine levels in urine and saliva are associated with exposure to tobacco smoke^{5,6} but do not distinguish reliably between smokers and non-smokers exposed to other people's smoke,⁷ and, because of the short half-life of nicotine in blood, they reflect exposure to smoke for the previous few hours only. There are implications for biochemical epidemiology in our results. In studies in which urine samples have been frozen, diseases which might be related to breathing other people's smoke can be investigated by measuring cotinine concentrations in samples from non-smokers who have, for example, lung cancer and in samples from matched non-smoking controls.

We thank Mrs Dora Bull for computing assistance and Mr P. A. Thompson for assistance with data coordination and sample collection. We also thank the Medical Research Council for financial support.

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FAILURE TO DETECT AGE-RELATED INCREASE OF NON-PATHOLOGICAL AUTOANTIBODIES

SIR,—It is a tenet of immunological theory that the immune system preserves a capacity for self-recognition; collapse of self-recognition may result in autoimmune disease. The idea that autoantibodies appear with increasing frequency with age, without necessarily any pathological association, has also gained general acceptance.¹

We have developed a new immunoassay procedure called "multi-dot immunobinding".² Antigens are applied to nitrocellulose in the form of dots, and bound antibody is revealed by an indirect immunoperoxidase test. The stain intensity of the dot is then a measure of the antibody titre. The method is simple to do and well suited to analyses where complete immunological profiles are required. Any combination of dots may be used on one strip of nitrocellulose. We have explored the applicability of this methodology for autoantibody profiles.³ While evaluating this method we accumulated a file of profiles for various groups of healthy individuals and patients. To our surprise there was no age-related increase in autoimmunity.

The sera came from healthy people, from patients with miscellaneous diseases irrelevant to autoimmunity, and from patients with rheumatoid arthritis and miscellaneous connective tissue diseases. IgG and IgM specific profiles consisted of assays for anti-native DNA, anti-denatured DNA, rheumatoid factor, beta cells, and various subcellular fractions derived from hela cells (nuclei, mitochondria, ribosomes, cytosol, nuclei, and nuclear RNP). Only data for anti-DNA and rheumatoid factor are given here (the other data did not lead to any different conclusion).

Table 1 shows the mean titres obtained by reflectance densitometry for healthy people and patients with no known autoimmune condition. There is no simple relation between mean titres and age. The validity of the assay system is supported by the raised titres found in patients with connective tissue diseases

TABLE 1—MEAN (±SD) OF AUTOANTIBODY TITRES* IN DIFFERENT AGE GROUPS

Age	No.	IgG anti-		IgM anti-		IgM-RF
		mDNA	dsDNA	mDNA	dsDNA	
Healthy + irrelevant diseases						
<30	10	2.9±1.4	0.5±1.3	15.3±15.9	1.2±2.0	5.5±6.1
30-39	12	3.1±1.4	2.4±4.1	9.2±5.1	0.9±1.0	4.2±2.6
40-49	10	3.4±2.9	1.0±1.1	4.6±4.3	0.8±1.3	3.5±2.8
50-59	40	2.6±4.5	0.5±0.9	5.3±10.1	0.7±0.9	4.0±3.0
60-69	12	2.4±2.0	1.3±3.2	3.1±3.8	1.3±1.9	3.0±3.9
>70	21	3.4±7.2	0.6±0.8	0.6±2.0	1.7±2.5	0.3±2.2
Connective tissue diseases						
54†	64	15.5±26.2	6.1±13.5	11.8±22.5	3.8±9.1	41.4±48.9

*m = single stranded and ds = double stranded. RF = rheumatoid factor

†Titres as demonstrated by absorbance units

† Mean age

TABLE 2—CORRELATION COEFFICIENTS OF AUTOANTIBODY TITRES
WITH RESPECT TO AGE

Group	No.	IgG anti-		IgM anti-		IgM-RF
		mDNA	dsDNA	mDNA	dsDNA	
Healthy + irrelevant diseases	104	-0.09	-0.16	-0.27	0.05	-0.01
	49	0.03	0.05	0.07	0.16	0.16

(bottom line of table 1). Age-related changes were not being masked by the combination of data from healthy individuals with data from patients with non-autoimmune diseases, as the correlations in table 2 show. Nor did any age-related increase in titre emerge when males and females were separately analysed.

We had thought that increasing symptomless autoimmunity with respect to age was well-established. However, there may be no conflict between our data and earlier findings. Perhaps the "forbidden" clones that accumulate with age are of restricted specificity and are not detected by all methods: use of our highly specific new method may then decrease the probability of detecting antibodies from such forbidden clones.

We know of two other studies in which age-related increases in rheumatoid factor (passive haemagglutination technique)⁴ and antinuclear antibodies⁵ were not found. These findings, with ours, raise the question of whether an increasing frequency of autoantibodies with age represents true autoimmunity or merely an increase in antibodies which happen to cross-react with some autoantigen(s).

Our results do not question the increasing frequency of autoimmune disease with age. In practice they mean that, when establishing normal values of autoantibody titres with the multi-dot immunobinding assay system, correction for age is not necessary.

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PLATELET ALPHA GRANULE PROTEINS IN STROKE AND TRANSIENT ISCHAEMIC ATTACKS

SIR,—Data on platelet activation in transient ischaemic attacks (TIAs) and stroke are confusing. Platelets when activated release both platelet factor 4 (PF4) and β -thromboglobulin (β TG), and great importance has been attached to measuring these two proteins in the plasma: de Boer et al¹ measured the plasma β TG in patients within

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Cotinine Analytical Workshop Report: Consideration of Analytical Methods for Determining Cotinine In Human Body Fluids as a Measure of Passive Exposure to Tobacco Smoke*

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A two-day technical workshop was convened November 10-11, 1986, to discuss analytical approaches for determining trace amounts of cotinine in human body fluids resulting from passive exposure to environmental tobacco smoke (ETS). The workshop, jointly sponsored by the U.S. Environmental Protection Agency and Centers for Disease Control, was attended by scientists with expertise in cotinine analytical methodology and/or conduct of human monitoring studies related to ETS. The workshop format included technical presentations, separate panel discussions on chromatography and immunoassay analytical approaches, and group discussions related to the quality assurance/quality control aspects of future monitoring programs. This report presents a consensus of opinion on general issues before the workshop panel participants and also a detailed comparison of several analytical approaches being used by the various represented laboratories. The salient features of the chromatography and immunoassay analytical methods are discussed separately.

Introduction

Environmental tobacco smoke (ETS) has increasingly become a health concern since a series of epidemiological studies between 1981 and 1986 (1-6)

reported an association between tobacco smoke exposure and increased risk of human lung cancer. Humble and co-workers (7) recently confirmed the health risk conclusions of earlier researchers and reported that people who never smoked and were married to smokers had about a 2-fold increased risk of lung cancer.

Methods for determining the degree of exposure of individuals has received much attention in recent years, and various biological markers have been studied as surrogate analytes for determining exposures.

A general consensus is that the nicotine metabolite, cotinine, is the most reliable and practical marker for exposure to tobacco smoke. Over the past several years a number of procedures have been reported for determining cotinine in human body fluids. The majority of these procedures use either a chromatographic technique or some form of immunoassay analysis.

This paper is a report from the two-day Cotinine Analytical Workshop, which was attended by invited health scientists and analytical chemists recognized for their expertise in studies of population exposure

*Chairperson: J. Lewtas, US EPA, Research Triangle Park, NC; Session chairpersons: F. Sperto, CDC, Atlanta, GA, R. Watts, US EPA, Research Triangle Park, NC; invited speaker/panel participants included: Neal Benowitz and Peyton Jacob, San Francisco General Medical Center; Colin Feyerabend, New Cross Hospital, London, England; Nancy Haley, American Health Foundation; George Knight, Foundation for Blood Research; Richard Kornfeld, Battelle Columbus Laboratories; John Langone, Baylor College of Medicine; Peter McElroy, Rosewell Park Memorial Institute; M. A. H. Russell, Maudsley Hospital, London, England; Karl Verebey, New York State Division of Substance Abuse Services; and Helen Van Vunakis, Brandeis University.

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to ETS and/or analytical methodology related to these studies. The workshop was jointly sponsored by the U.S. Environmental Protection Agency (EPA) and Centers for Disease Control (CDC) and was attended by 32 scientists who shared their expertise in immunoassay or chromatography methods for cotinine and provided guidance for developing and establishing related programs for determining passive exposures to tobacco smoke. The meeting objective was to compare the various analytical approaches to cotinine analysis and to make recommendations regarding the general aspects of establishing and conducting monitoring programs. Discussions included quality assurance/quality control (QA/QC) programs to support cotinine monitoring studies and also the possibility of conducting a future interlaboratory methods comparison study. The diverse analytical approaches represented by chromatography and immunoassay methods for cotinine were separately discussed and reported by respective work groups. The purpose of this communication is to summarize discussions from the immunoassay and chromatography work groups relevant to the aforementioned topics and to convey the workshop general consensus on other joint issues including QA/QC aspects of ETS studies.

Chromatography Group Report

The workshop participants with expertise in developing and applying chromatography methods for determining cotinine in biological fluids met in a one-day session. The goal of this session was to develop a group consensus on several key issues including a) general method considerations and approaches, b) QA/QC programs to support cotinine monitoring studies, and c) considerations related to conducting an interlaboratory methods comparison study. The following is a summary of the chromatography group discussions and a draft of their recommendations related to topics a and c. The QA/QC recommendations are contained in a separate section.

General Method Considerations

Sample Type. The body fluids discussed for monitoring tobacco smoke exposure included blood serum, saliva, and urine. Group consensus was that all three are generally acceptable; however, the choice of a body fluid to analyze should be predicated on the goals of the specific monitoring program. For studies that require a quantitative assessment of exposure, blood was recommended by the group as the fluid of choice (8). Saliva was also considered acceptable, and good correlations were reported between saliva and blood for results from the same subject (9). Sample collection considerations, however, resulted in the selection of blood as the sample medium of first choice. Analysis of either blood or saliva for cotinine permits an estimate of the degree of exposure to tobacco smoke in persons passively exposed at home or in the work place. While

cotinine determination in urine was also recommended for estimating exposure, it was generally felt that estimation based on urinary cotinine excretion would be less reliable than estimation based on plasma or salivary levels. Cotinine excretion is variable across and within individuals depending on renal function, urine flow rate, and urine pH (10). Urine results may be expressed as micrograms of cotinine per milligram of creatinine in order to correct, in part, for the variable dilution effects. This correction or normalization, however, introduces additional variability since this requires another analytical determination (and opportunity for experimental error), and creatinine excretion rates for individuals are also variable. Horstmann (11) reported creatinine excretion rate for 56 subjects to be 1.11 ± 0.68 g/day (mean \pm SD). Hoffman and Brunneman (12) also found 13 nonsmokers on a controlled diet to have creatinine values of 1.65 ± 0.5 g per 24 hr urine (mean \pm SD). The coefficients of variation between subjects for these two studies were 61 and 30%, respectively.

Sample Collection and Handling. Chromatography procedures for cotinine generally require analysis of a 1 mL sample with an additional 1 mL volume needed for reanalysis. A total sample volume of 2.5 to 3.0 mL was therefore recommended. Glass and/or polypropylene sample tubes with screw cap closures were recommended. The polypropylene tubes were preferred to avoid breakage during shipment. Minimum size sample tubes were suggested to reduce volume losses from freeze drying during long-term storage.

Blood should be centrifuged at the field site and the serum samples frozen prior to shipment to the laboratory. Urine should be frozen soon after collection to prevent bacterial degradation of the sample. Saliva may be collected by expectoration into a sample tube; however, an alternative saliva collection procedure that uses highly adsorbent dental rolls is recommended (13). The subject is asked to place a dental roll in the mouth for approximately 15 min. The sample is then placed in a tube and frozen prior to shipment to the laboratory. The thawed sample is regenerated at the laboratory by placing the dental roll in a glass syringe and compressing with a glass plunger. The resultant clear liquid may then be aliquoted for analysis.

Shipment in a frozen condition with dry ice was recommended for all three sample types to prevent bacterial degradation of the sample matrix. Loss or degradation of the cotinine analyte was not considered to be a problem since participants had found this compound to be stable.

Upon receipt at the laboratory, samples should be placed in a freezer (approximately -20°C) until analyzed. Samples that will be held in excess of one year should be stored at -80°C . No cotinine degradation problems were reported for frozen samples. Precautions were recommended, however, to prevent concentration errors resulting from freeze-drying of samples stored over one year in a frost-free freezer.

Analytical Method Considerations

The group consensus was that the analytical method should permit the determination of nicotine and cotinine in a single analysis and should allow a clear separation and distinction between these and other analytes that may be present. The method should be sufficiently sensitive to give good definition of passive exposure and thereby yield analytical results which will show a distinction, for example, between a child or other nonsmoker that is exposed in the home and one that is not. Tables 1 and 2 list the range of detection limits for both chromatography and immunoassay methods.

The importance of this sensitivity consideration was supported by the 1981 report of Hirayama (1) and the 1987 report of Humble et al. (7) which showed an increased risk of lung cancer for a spouse exposed to a smoker in the home. Russell reported that cotinine levels in children's saliva averaged 0.44 ± 0.68 ng/mL where no parents are smokers, 1.31 ± 1.21 ng/mL where only the father smoked, 1.95 ± 1.71 where only the mother smoked, and 3.38 ± 2.45 ng/mL where both parents are smokers (13). This study used an analytical method with a detection and quantification limit of 0.1 ng/mL, which permitted classification of the lowest exposures into exposure distributions differing by only 0.1 ng/mL. Over 30% of the children from nonsmoking homes had cotinine concentrations below the 0.1 ng/mL detection limit. In the groups where one or more parents smoked, the cotinines were significantly ($p < 0.01$) elevated, and 50% of the children of the lowest exposed group had less than 1 ng/mL (when only the father smoked). Table 1 shows that several available chromatography methods have detection limits ranging from 0.1 to 0.2 ng/mL while the most sensitive immunoassay method in Table 2 reports a 0.3 ng/mL detection limit.

The question of analyte volatility losses during analysis was discussed, and it was generally agreed that if nicotine were included as an analyte, precautions would need to be taken to prevent loss during concentration steps. Acidification to convert nicotine to a salt form prevents losses during concentration.

Cotinine primary standards are used in the free base form by some analysts; however, a salt form was preferred by meeting participants, since the free base form is hygroscopic and difficult to maintain at a well-defined purity. A perchlorate salt of cotinine was recommended for preparation of 1 mg/mL stock solutions in 0.01 N HCl (8). This standard solution could be frozen and kept indefinitely. The group consensus was that a salt form of cotinine should be made available as a primary standard.

Chemical analysis is usually accomplished by gas chromatography with nitrogen/phosphorus thermionic detection (GC-NPD) or GC-mass spectrometry (GC-MS) using either electron impact ionization or

chemical ionization. Packed columns for GC were successfully used; however, fused silica capillary columns containing a methyl silicone or methyl phenyl silicone liquid phase were recommended (see Table 1 HRGC references).

A high-performance liquid chromatography (HPLC) method using a C_{18} reversed phase column with paired ion chromatography and UV detection (at 257 nm) was also reported by McElroy where the HPLC method of Machacek and Jiang (14) was modified for analyzing urine samples at passive exposure levels. Further improvement in HPLC sensitivity and detection limit is required before application to the more limited sample volumes generally available for blood or saliva. HPLC was considered a very promising approach due to the highly efficient columns now available and the stability and reproducibility of response commonly obtainable by UV detection.

The final quantitation of residues in all methods was accomplished with internal standards and standard curves developed from fortified blank samples. It was recommended that standard curves be prepared daily or with each batch of samples. A variety of internal standards were used ranging from deuterated cotinine and nicotine for GC-MS to chemically similar compounds for other GC or LC detectors.

Table 1 lists the chromatography methods (14-17) presented at the workshop and summarizes the salient features of each. Information for this table was derived from questionnaire responses submitted by each author/participant.

Chromatography Group Recommendations

The chromatography group recommended that an interlaboratory methods comparison study be conducted prior to any large-scale monitoring efforts aimed at determining population exposure to tobacco smoke. Specific suggestions and recommendations relating to method comparison studies were as follows:

- Separate studies should be conducted for passive exposure levels and active smoker levels.
- Statisticians should be used in planning study samples.
- Blood, urine, and saliva should be included in each study.
- Immunoassay and chromatography methods should be included in each method comparison study.
- Samples should be fluids from exposed individuals and also from fortified blanks in order to look for bias from chromatography or immunoassay methods through measurement of artifacts or metabolites related to nicotine/cotinine.
- The study coordinator should supply standard reference material(s) to each participating laboratory.

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Table 1. Summary of passive exposure chromatography methods.

	Machacek and Jiang (14)	Jacob, et al. (15)	Verebey et al. (16)	Feyerabend et al. (17)	Kornfeld (personal communication)
Sample type	Urine	Blood, plasma, urine	Serum	All biological fluids	Urine
Vol. analyzed, mL	6	1	0.5	1	5
Concentration step	N ₂ evaporated to dryness	N ₂ evaporated to dryness	None	N ₂ evaporated to dryness	N ₂ evaporated solvent exchange
Extraction method	SPE column Chloroform elution	Solvent extraction	Solvent extraction	Solvent extraction	Solvent extraction
Isolation step	Acid/base partition	Acid/base partition	Acid/base partition	None: plasma and saliva back extract urine	None
Determination	HPLC reverse-phase Paired ion chromatography	HRGC-NPD HRGC-MS	HRGC-NPD	GC-NPD	HRGC-MS
Quantitation	Internal standard Calibration curve	Internal standard Calibration curve	Internal standard Calibration curve	Internal standard Calibration curve	Internal standard Calibration curve
Linear range	0-500 ng	0-4000 ng/mL	40-400 ng/mL	0-15,000 ng/mL	1-500 ng/mL
Detection limit	<1 ng/mL	0.2 ng/mL	5 ng/mL	0.1 ng/mL	0.13 ng/mL
Quantification limit	1 ng/mL	0.5-5 ng/mL	5 ng/mL	0.1 ng/mL	0.25 ng/mL
CV, %	13	6.8 (3.0 ng/mL) 12 (1.0 ng/mL) 13 (0.5 ng/mL)	5.6	7.7	6
% recovery	90	107	90	90	85

Immunoassay Group Report

Participants in the workshop with expertise in the development and use of immunoassays for detecting cotinine in biological fluids met independently of the chromatography group to discuss and make recommendations regarding methodology and applications of immunoassay in monitoring passive as well as active exposure to tobacco smoke, QA/QC programs, and interlaboratory methods comparison. The following discussion presents an overview of the available immunoassay techniques for cotinine analysis, their applications with advantages and disadvantages, and the views and recommendations of the immunoassay panel members. There is notable agreement between this group and the chromatography group on most common issues outside the technical aspects specific to each methodology.

General Method Considerations

Introduction. The first radioimmunoassay (RIA) for cotinine was reported in 1973 (18,19). Antisera were raised in rabbits and goats immunized with a covalent conjugate prepared by linking cotinine 4'-carboxylic acid to immunogenic carrier proteins, such as bovine serum albumin and keyhole limpet hemocyanin. The radioactive tracer was prepared by labeling a tyramine derivative of cotinine 4'-carboxylic acid with ¹²⁵I; since then, [³H]cotinine has been prepared enzymatically (19) from [³H]nicotine and is now widely used. Another approach uses cotinine derivatized at the 1-position in the pyridine ring for preparing the immunogen and as a precursor of an ¹²⁵I-labeled tracer (20). The original assay has been used to measure cotinine levels in physiological fluids, e.g., urine, blood, saliva, amniotic fluid, and spinal fluid

Table 2. Summary of immunoassay methods.

	Langone et al. (18) Langone and Van Vunakis (19)	Haley et al. (22)	Knight et al. (23)	Bjercke et al. (27,28)
Sample type and volume analyzed, mL	Urine (0.02-0.05) Serum (up to 0.5) Saliva (0.02)	Urine, plasma, saliva (0.005-0.025)	Urine (0.01) Serum (0.1)	Urine, serum, saliva (0.1 in RIA; 0.01 in microtiter plate assays)
Assay type	RIA (¹²⁵ I, ³ H)	RIA (³ H)	RIA (¹²⁵ I)	RIA (¹²⁵ I, ³ H), ELISA,* FIA
Quantitation	Internal standard Calibration curve	Internal standard Calibration curve	Internal standard Calibration curve	Internal standard Calibration curve
Detection limit, ng/mL	2	0.37	0.3	0.5-1.5
Quantitation limit, ng/mL	2	1	1	0.5-1.5
CV, %	6-10	10	10-15	9-14

*Enzyme-labeled protein A cannot be used to assay concentrated serum.

(18,19,21-23) of active smokers and serum, urine, and saliva (24-26), of passive smokers.

More recently, monoclonal antibodies specific for cotinine have been prepared and used to develop fluid phase RIAs with the ^{125}I - and ^3H -labeled tracers as well as enzyme-linked immunosorbent assays (ELISA) and a fluorescence immunoassay (FIA) in a microtiter plate format (27,28). These assays also have been used to measure cotinine levels in fluids of active (27,28) and passive smokers (Langone et al., unpublished results).

Test Samples and Standards. Because the original RIAs and the monoclonal antibody-based nonisotopic assays have been developed for analysis of unextracted physiological fluids, careful attention must be paid to possible nonspecific inhibition of antigen-antibody binding resulting from effects of pH or high concentrations of salts or urea, e.g., in urine. In this regard the immunoassay group agreed and strongly recommended that pooled standard samples of serum, saliva, and urine containing known amounts of added or endogenous cotinine should be made available through an agency such as the National Institute of Standards and Technology. There was general agreement that GC/MS would be the best method to establish the cotinine concentration for purposes of methods comparison and that levels should cover the range from cotinine-free through concentrations found in passive and active smokers. Essentially cotinine-free samples might be collected from a population that would represent a group with minimal exposure to tobacco smoke (e.g., Mormons in Utah). The suggestion also was made that low-level or essentially cotinine-free fluids might be treated (e.g., by absorption with XAD-2 resin or charcoal) to remove possible traces of cotinine. However, because absorption could remove other constituents that might affect the assays, it was not considered to be a firm recommendation.

Although it was suggested that urine may be the fluid of choice for RIA analysis, there was no strong consensus for priority over serum or saliva. In this regard, one participant pointed out the advantage that salivary cotinine levels determined by RIA are independent of saliva flow (Van Vunakis and Regas, unpublished results). The monoclonal antibody assays also have been used to detect cotinine in saliva and urine of passively exposed children (Langone et al., unpublished observations), and these investigators tended to favor the use of saliva. In addition to the use of dental rolls as discussed by the chromatography group, one member of the immunoassay group suggested that subjects chew a piece of Teflon tape to stimulate the flow of saliva that is then collected in a glass vial. It was pointed out that Teflon will not contaminate the sample. Regardless of which fluid is tested, it was recommended that samples be centrifuged (e.g., 2000g for 10-20 min or 10000g for 1-2 min) to sediment particulate matter before analysis. Immunoassay group participants concurred with the

sample handling recommendations given in the Chromatography Group Report.

Comparison of the Assays

The original RIA and variations of it are used by the immunoassay group participants. Therefore, the discussion focused on this method and the monoclonal antibody based assays, the salient features of which are summarized in Table 2.

Reagents. The same immunogen was used to produce the rabbit, goat, or sheep antisera and the monoclonal antibodies. However, it was emphasized that cotinine 4'-carboxylic acid (and the ^{125}I -labeled derivative) is a mixture of stereoisomers giving rise to a heterogeneous population of polyclonal antibodies recognizing both natural (-)-cotinine and the (+)-enantiomer. Also, conventional antisera contain a population of antibodies that bind specifically to the linkage group that joins cotinine to the immunogenic carrier protein and to the tyramine group in the ^{125}I -labeled derivative. The practical consequences are a relatively shallow standard inhibition curve and the failure to achieve 100% inhibition of immune binding with (-)-cotinine. Although these problems are circumvented by using (-)- ^3H cotinine, this assay is somewhat less sensitive, owing to the lower specific activity and counting efficiency achieved with tritium. Also, disposing of large volumes of radioactive scintillation fluid is a major concern.

Two approaches have been used with some success to improve the quality of the ^{125}I -RIA with rabbit antisera. They involve removing antibody group antibodies by absorption with a nicotine-hemocyanin conjugate (29) and preparing an ^{125}I -labeled cotinine derivative with a bridging group different from that present in the immunogen (30). In contrast, monoclonal antibodies to cotinine were produced in a way that avoided the problems inherent in the use of polyclonal antisera (27,28). Although the immunogen contained a mixture of isomers, the hybridomas were screened using (-)- ^3H cotinine to optimize chances of detecting antibodies that preferentially recognize the naturally occurring isomer, but not the bridging group in the immunogen. Furthermore, it was pointed out that monoclonal antibodies are preferred standard reagents for immunoassay because they are continuously available and are homogeneous in terms of binding affinity and specificity.

The specificity of any newly produced antiserum must be fully characterized one time with a battery of compounds that would include at least cotinine, nicotine, and metabolites such as nicotine N'-oxide, nor-nicotine, and *trans*-3'-hydroxycotinine. This recommendation holds even for new antisera prepared with a proven immunogen, since the response of individual immunized animals cannot be predicted. However, all agreed that when the properties of the antiserum had been established, it was unnecessary for each laboratory that received that antiserum to

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complete a thorough reexamination of specificity, although it would be good laboratory practice to routinely compare the cotinine and nicotine inhibition curves.

Immunoassay methods have often reported using standard cotinine as the free base. However, because cotinine is hygroscopic and difficult to weigh accurately, all participants agreed that a nonhygroscopic salt of cotinine, such as the perchlorate or fumarate, would be the preferred standard.

Assay Performance. In the original RIA, antibody-bound and free-labeled cotinine were separated by the double-antibody method in which a heterologous antibody directed against the species of anticotinine was used to precipitate antigen-antibody complexes (18). Other techniques can be used including precipitation with ammonium sulfate or polyethylene glycol (29). Although the latter methods are faster and less expensive, there was some concern expressed that background radioactivity precipitated by ammonium sulfate, when normal serum is used in place of anti-cotinine, can exceed 10% of the added amount of cotinine tracer.

In contrast to the conventional fluid phase RIAs, the monoclonal antibody-based assays are carried out in a solid-phase system in which a cotinine-polylysine conjugate is passively adsorbed to the surface of 96-well plastic microtiter plates (27,28). Immobilized cotinine and fluid phase cotinine in the test sample compete for monoclonal anticotinine, which is detected with a variety of enzyme-labeled antiimmunoglobulin reagents including the bacterial product, protein A. Assay sensitivity can be enhanced by using a sandwich procedure in which rabbit anti-mouse immunoglobulin is added before (or along with) labeled protein A. It was emphasized that protein A reagents cannot be used to detect low levels of serum cotinine, because host IgG will bind nonspecifically to the microtiter wells giving high background binding of the enzyme-labeled protein A tracer.

Compared to times when rabbit antisera were used, assays with monoclonal antibodies were more sensitive, the standard curves were steeper, and the antigen-antibody reaction was completely inhibited by (-)-cotinine, even when the ^{125}I -labeled tyramine derivative was used in RIA (28). There was good agreement between the levels of cotinine found in saliva and serum of smokers determined by conventional RIA, the monoclonal antibody ELISA and GC (27,28). It was pointed out that high quality rabbit antisera also can be used in the solid phase nonisotopic immunoassays with titers that can be 100- to 1000-fold higher than in RIA (27,28).

Specificity and Sensitivity. Both polyclonal and monoclonal antibodies are specific for cotinine (18,19,27). Approximately 50 to 100 compounds that have been tested in the immunoassays including several nicotine metabolites, related tobacco alkaloids, and other compounds that retain structural features of either or both ring systems found in nicotine or

cotinine fail to inhibit the antigen-antibody reactions at levels that would interfere in the assays.

One participant emphasized that literally thousands of serum and urine samples from both active smokers and nonsmokers had been analyzed over a period of several years and that few, if any, false positives had been reported. Although the subjects studied are mainly from the U.S. and England, these data support the view that diet or other factors such as prescription or other drugs do not interfere in the assays and are consistent with high specificity of anticotinine. It was pointed out that differences in diet or drug use must be considered when other populations are studied, or at least be aware that interference in the assays might arise from factors which have not appeared to date.

The immunoassays generally can detect cotinine down to the ng/mL level or less (Table 2), although it was emphasized that sensitivity can be affected by the need to dilute samples (e.g., urine) that may give spurious results when higher concentrations are tested. This point was discussed at some length with the participants in agreement that a sensitivity for cotinine of 0.1 ng/mL of physiological fluids could not generally be achieved with confidence using the available immunoassays. In this regard, it was pointed out that differences in sensitivity limits between chromatography and immunoassay likely reflect fundamental differences in methodology and are not strictly comparable. GC methods, for example, might extract and analyze a considerably larger portion of sample than would be analyzed by immunoassay.

Analytical Results. There was general agreement that cotinine concentration should be expressed as nanogram per milliliter. However, urine values also should be given as nanogram per milligram creatinine, as this ratio is used conventionally in the medical literature to account for differences in urine volume. Because low levels of creatinine in infants relative to adults may result in misleading values that fall into the range reported for active smokers, the need to include primary data for urine was stressed. Furthermore, experience has shown that urinary cotinine levels determined by conventional RIA generally are 30 to 50% higher than values obtained for the same samples by GC. Discussion centered on the possibility that the higher RIA values may reflect cross-reactivity of anti-cotinine with *trans*-3'-hydroxycotinine, which recently has been reported to be a major nicotine metabolite found in smokers' urine at levels up to three times higher than cotinine (31).

Since this meeting, synthetic *trans*-3'-hydroxycotinine (supplied by Dr. Peyton Jacob, San Francisco General Medical Center) has been shown to cross-react only 1 to 2% compared to cotinine in the monoclonal antibody based ELISA; one participant stated that he found only about 5% cross-reactivity with his rabbit antiserum in RIA. This degree of cross-reaction would not account for the discrepancy

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Table 3. Summary comparison of chromatography and RIA methods.

	Chromatography methods	RIA methods
Sample type	Blood, saliva, urine	Blood, saliva, urine
Vol. analyzed, mL	0.5-6	0.005-0.5
Extraction and concentration	Yes	No
Quantification	Internal standard Calibration curve	Internal standard Calibration curve
Detection limit, ng/mL	0.1-5	0.3-2
Quantification limit, ng/mL	0.1-5	0.5-2
CV, %	5.6-13	6-15

in the urine values, and it was agreed that further research was needed to clarify the basis for the differences.

Considerations in Selecting an Analytical Technique

Table 3 shows some comparisons for RIA and chromatography methods. Apparent differences are in sample volumes used, sample work-up requirements, and limits of detection. RIA methods use less than 10% of the sample volumes required for chromatography methods, and this is a major reason that RIA detection limits are not as low as those for chromatography methods. Because RIA methods do not require sample manipulations such as extraction and concentration, they are faster, simpler, and presumably less expensive. Chromatography procedures not only have the advantage of increased sensitivities, but also are more specific and can provide quantification of both nicotine and cotinine in a single analysis. Workshop participants agreed that the choice between these two approaches would depend on the goals of a particular study. Both approaches have been found to be 100% effective in discriminating smokers from nonsmokers (32). This particular goal would favor the use of an RIA method. At least one participant suggested that the more sensitive chromatography methods are recommended to characterize ETS exposures for plasma or saliva concentrations when levels are less than 1 ng/mL.

A compilation of literature values for cotinine concentrations in body fluids of nonsmokers before and after documented ETS exposures is shown in Table 4. This comparison indicates a similarity between plasma and saliva concentrations, while urine values are often a factor of two or more higher. This is a primary reason that urine is often the fluid of choice when RIA methods are used in passive smoking studies.

Quality Assurance for Laboratories Assaying Cotinine

Participants in the cotinine workshop discussed the need for developing a quality assurance (QA) program for monitoring performance of laboratories

assaying cotinine for the purpose of assessing exposure to ETS. When assuming many subjects, such a QA program would be essential to ensure that the conclusions reached are based on reliable data. A one-time exercise where the ability of laboratories to measure cotinine levels found in both active smoking and for passive exposure to ETS was considered as an alternative possibility. This suggestion was prompted by the realization that although published data on cotinine levels found in body fluids for active smokers show reasonable agreement, levels of cotinine reported for subjects exposed to ETS show considerable variation. Such differences might not be unexpected when measuring the low levels of cotinine found in ETS exposure, given that the detection limits for existing analytical methods approximate these cotinine levels.

To evaluate the between-laboratory variation in cotinine analyses, an international study was initiated by the Forschungsgesellschaft Rauchen und Gesundheit mBH in Hamburg (32). Eleven laboratories experienced in measuring nicotine and cotinine by RIA and/or GC participated. Serum and urine specimens from eight nonsmokers and eight smokers, and from two nonexposed nonsmokers spiked with nicotine and cotinine were distributed on dry ice to each laboratory. Results were returned and analyzed by method and by laboratory. Recoveries on both the urine and serum specimens spiked with cotinine corresponding to levels found in smokers ranged from 79 to 119%, with the exception of one laboratory with a 20% recovery (the data from this laboratory were excluded from further analysis). The interlaboratory coefficient of variation on these same samples was excellent (9-13%). The coefficient of variation on samples from smokers was fairly large, however, ranging from 18 to 45% for serum and 21 to 59% for urine. Further, cotinine levels reported for urine were about 60% higher than from those using RIA as compared to GC, suggesting a possible interfering substance in the immunoassay system. Cotinine levels reported for nonsmokers were extremely variable, and a number of laboratories could not detect cotinine in serum from exposed nonsmokers. In addition, cotinine values reported by some laboratories bore no relationship to estimated ETS exposure, or they were so high as to be unrealistic. In spite of this variability, all laboratories were able to discriminate smokers from nonsmokers with 100% effectiveness.

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Table 4. Mean or median concentrations of cotinine in nonsmokers before and after exposure to environmental tobacco smoke.

Study reference	Plasma cotinine, ng/mL		Saliva cotinine, ng/mL		Urine cotinine, ng/mL	
	Before	After	Before	After	Before	After
(33)	0.82	2.04	0.73	2.48	1.55	7.71
(34)	1.1	7.3	1.5	8.0	4.8	12.9
(9)	0.8	1.8-2.5	0.7	2.2-2.8	1.5	6.5-9.4
(13)			0.4	1.3-3.4		
(35)	0.9-1.7	2.6-3.3	1.0-1.7	1.4-2.5	14	21-55
(36)					8.5	25.2
(25)						2.8-29.6
(37)			1.3-1.7	2.4-5.6		

The cotinine results reported for ETS exposure should be viewed with caution, however. A number of the participants at this conference workshop, who also were in the study, indicated that the volumes provided were insufficient for repeat analysis using GC or an assay was used which had not been optimized for measuring passive levels of cotinine. A further limitation of the study was that recovery of spiked cotinine was only assessed for smoking levels. Finally, immunoassays based on monoclonal antibodies were not included, nor were HPLC methods evaluated.

This interlaboratory study indicates the need for further information on the reliability of data provided by laboratories for study subjects exposed to ETS. A quality assurance program could provide such information, as well as an ongoing assessment of quality and a mechanism for improving performance.

QA Recommendations

Interlaboratory QA. The need for an interlaboratory quality assurance program was endorsed by most of the session participants, with some concern being expressed that the number of samples evaluated be kept to reasonable limits to minimize unnecessary assays. It was recommended that such a program should be administered by a QA coordinator laboratory. The coordinating laboratory would be responsible for monitoring the performance of participating laboratories and for providing specified samples as standards and/or controls. This laboratory should have in-house expertise or have access to laboratories having expertise in both immunoassay and high resolution gas chromatography/mass spectrometry (HRGC-MS).

Suggested objectives of the QA coordinator laboratory include:

- To provide an objective measure of the precision and accuracy of analytical methods used routinely by laboratories assaying cotinine.
- To identify preferred method(s) for measuring cotinine.
- To assess the reliability of results provided by different laboratories.

- To provide a mechanism for improving performance through knowledge of the performance of others.
- To serve as a resource center for communication and exchange of information among participants.

Recommended mechanisms for accomplishing the foregoing objectives are as follows:

Interlaboratory Quality Assurance Studies: Quality Assurance Samples. The coordinator laboratory should periodically conduct a blind or check sample study consisting of authentic biological fluids (serum, urine, or saliva) with actual or spiked levels of cotinine. Samples should be selected to represent cotinine levels typical of those found in passive and active smoking. Authentic biological samples with actual levels of cotinine are strongly recommended because only they will contain nicotine metabolites or other substances that may interfere in assays. In addition, blank samples spiked with known levels of cotinine should be distributed to evaluate recovery. Finally, samples with high levels of cotinine should be diluted with negative specimens to check for linearity. Samples should ideally have target values assigned by the QA coordinating laboratory through use of reference methods. Data returned by participants would be analyzed and reports containing results and a critique distributed.

Field Study Samples. The QC coordinating laboratory may assist organizations carrying out field studies in assessing the performance of the study laboratory on actual study subjects. The workshop considered that this could be accomplished by submission at intervals of blind duplicates: duplicates of the same study subject submitted at intervals to assess precision; split samples: sample is split with one portion being sent for analysis to the study laboratory and one portion to the QC coordinating laboratory for comparison purposes; blanks: samples that are considered free of analyte to serve as a check on environmental contamination.

Ancillary Activities of the QA Coordinating Laboratory: Primary Reference Standard(s). A strong consensus was reached that a well-characterized, pure, primary reference standard be made available. This material should be aliquoted into quantities sufficient to allow any laboratory to use

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the standard for assay calibration. Handling and storage information should also be provided along with suggested methods for preparing secondary standards. It was generally agreed that cotinine should be in the form of a salt, since cotinine freebase is hygroscopic and, therefore, likely to vary in composition dependent on handling conditions. The perchlorate salt was suggested as one possibility (see chromatography group report). It was further recommended that the standard be supplied in solution to preclude errors due to dilution. The GC group made the suggestion that the National Institute of Standards and Technology might be the appropriate agency to prepare such a standard. The QC coordinating laboratory could then distribute the standard.

Biological Reference Samples. The suggestion was made that, in addition to providing a primary reference standard, the QA coordinating laboratory make available authentic biological samples from actual smokers and subjects exposed to ETS. Cotinine concentrations would be established by the QA coordinating laboratory using a reference method(s) and declared on each reference sample. Such samples would be important because the cotinine would be present in the matrix (urine, serum, saliva) actually used by analysts, thus allowing evaluation of possible matrix interference. In addition, such specimens would contain nicotine, nicotine metabolites other than cotinine, and other substances which might interfere in the assay.

The GC group also felt that blank samples, i.e., those essentially free of cotinine would be desirable. Suggested sources were bovine serum or human samples with very low exposure to ETS. Pooled specimens might be necessary because obtaining sufficient volume of biological reference samples could prove difficult. Samples would therefore be provided in restricted quantities only to allow laboratories to periodically evaluate their own method.

Reference Method. Workshop participants also discussed a reference method for establishing cotinine levels in biological samples. The consensus of the group was that GC/MS would be the ultimate reference method because of its extreme specificity. However, in the group discussion, the GC group pointed out that although the method is highly specific, it ultimately is no better than the reliability of the extraction and evaporation methods chosen to prepare samples for analysis. A further concern was that differences in assigned values may result from differences attributable to the detection method (chemical ionization or electron impact). Consequently, it appears unlikely that a gold standard will be available and acceptance of a reference method will depend ultimately on judgment of its reliability. Representatives of the National Institute of Standards and Technology indicated that their practice is to evaluate a variety of independent methods, and if sufficient agreement is reached, a certified value is

provided, albeit with the understanding that confidence limits are somewhat uncertain. In the absence of agreement between various methods, NIST provides a consensus value(s) for informational purposes.

In the event that GC/MS is adopted as a reference method, the implication for immunoassayists is that their performance would be judged against this standard. Judging immunoassay results against GC/MS is not without precedent, since other immunoassays, such as those for steroids, are already compared to this method.

Postscript

A cotinine spiked, freeze-dried human urine reference material is being prepared by the National Institute of Standards and Technology (formerly the National Bureau of Standards). Three lots with different cotinine concentration levels are being prepared: a) an unspiked blank level (< 1 ppb), b) an approximately 50 ppb low level, and c) an approximately 500 ppb high level. This material (EPA/NIST Reference Material 8444) is planned for issue during the first quarter of 1989. The material may be ordered from: Office of Standard Reference Materials, Building 222 Room B-311, National Institute of Standards and Technology, Gaithersburg, MD 20899. Telephone 301-975-6776. Technical information may be obtained from Dr. Lane Sander, Organic Analytical Research Division, Center for Analytical Chemistry, NIST, Gaithersburg, MD 20899. Telephone 301-975-3117.

A cotinine perchlorate salt reference material is also being planned for development by NIST. A date has not been determined, however, for release of this standard.

The research described in this report has been reviewed by the Health Effects Research Laboratory, U.S. Environmental Protection Agency, and approved for publication. Approval does not signify that the contents necessarily reflect the views and policies of the Agency nor does mention of trade names or commercial products constitute endorsement or recommendation for use.

The previously listed speaker/panel participants are acknowledged for their workshop participation and vital contributions to this report. The authors are also appreciative of the attendance and active participation of the following university and government agency representatives: Larry Claxton, Ruth Etzel, Linda Forehand, George M. Goldstein, Elaine Gunter, Katharine Hammond, Ed Hu, Henry S. Kahn, Kevin J. Kimbrell, Dave Mage, Ronald K. Mitchum, Judy Mumford, Todd C. Pasley, Terry Pechacek, George Provenzano, D. W. Sepkovic, Wanda Whitfield, Ron Williams, Deborah Winn, Stephen A. Wise, and Ou-Li Wong.

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Simulation and evaluation of nicotine intake during passive smoking: Cotinine measurements in body fluids of nonsmokers given intravenous infusions of nicotine

The technique of monitoring cotinine concentrations in body fluids as a means of measuring nicotine intake during passive smoking has been evaluated in two studies, both of which used intravenous infusion to simulate nicotine intake. In the first study, nicotine and cotinine were given separately, for 1 hour in four different intravenous doses (3.2, 15.4, 30.9, and 61.7 nmol/min) to each nonsmoker. In the second study, nicotine and cotinine were infused for 4 hours; each subject received five different doses of nicotine (1.5, 3.1, 6.2, 10.8, and 15.4 nmol/min) and one of cotinine (10.8 nmol/min). The concentration of cotinine was constant in both plasma and saliva from 1 to 4 hours after the nicotine infusion; the plateau levels of cotinine were found to be linearly and directly related to the nicotine intake. The ratio of salivary to plasma cotinine was 1:1.27. A linear relationship was also found between nicotine and cotinine infusion rates and the AUC values for cotinine. The fraction metabolized to cotinine was found to be about 0.6. The results from these studies show that: (1) there is a linear relationship between the plateau concentration of cotinine and the amount of nicotine infused over a period of 1 up to 4 hours; (2) salivary cotinine provides the same information on nicotine intake as does plasma cotinine; and (3) single measurements of either plasma or salivary cotinine concentrations at 1 to 4 hours after the exposure could be used to predict the nicotine intake during 1 to 4 hours of environmental tobacco smoke exposure. (*CLIN PHARMACOL THER* 1990;47:42-9.)

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It has been suggested that the extent to which nonsmokers are exposed to environmental tobacco smoke can be estimated by measuring the concentrations of certain tobacco smoke constituents in body fluids of nonsmokers.¹ Nicotine, which is specific to tobacco, and cotinine, a major metabolite of nicotine, have been used for this purpose. Cotinine is considered to be the better marker of nicotine exposure in both smokers and nonsmokers² because its biologic half-life in body fluids is much longer than that of nicotine (approximately 15 hours compared with 2 hours).^{3,4} The concentrations of

cotinine are therefore less dependent on exact sampling times. Moreover, renal excretion of nicotine is influenced by urinary pH, whereas that of cotinine is hardly affected.

The estimation of nicotine exposure on the basis of cotinine levels in body fluids calls for knowledge of the dynamics of cotinine. Specifically, the fraction of nicotine that is converted to cotinine in the body needs to be known, as well as the rates of cotinine elimination. In addition, information is required about the quantitative relationship between nicotine intake and steady-state cotinine levels. Such data are available for nicotine and cotinine only for levels found in smokers,⁵ not for the lower concentrations in nonsmokers. We therefore undertook the present study, in which short-term exposure to environmental tobacco smoke was simulated by infusing small amounts of nicotine intravenously for periods of 1 and 4 hours. Nicotine and cotinine concentrations were measured in plasma,

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Received for publication April 11, 1989; accepted Aug. 28, 1989.
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13/1/16440

Table 1. Subject characteristics and dosing of nicotine and cotinine

Subject	Age (yr)	Body weight (kg)	Infusion* (1 hr)	Infusion* (4 hr)
1	33	64	N/C	N
2	37	83	N/C	N/C
3	34	78	N/C	
4	30	75	N/C	N/C
5	37	66	N	
6	52	78	N	
7	22	80	C	
8	31	70	C	
9	30	90		N
10	23	72		N/C
11	33	73		N/C
12	34	89		C
13	27	60		C

*N, Nicotine; C, cotinine.

saliva, and urine during and up to 4 hours after the termination of the infusions.

SUBJECTS AND METHODS

Two studies were performed, each comprising two sets of experiments. In study 1, nicotine and cotinine were administered separately by intravenous infusion at four different rates over a period of 1 hour. In study 2, nicotine and cotinine were again administered separately, but the infusion time was extended to 4 hours, and cotinine was infused at one and nicotine at five different rates.

Subjects

The subjects were thirteen healthy nonsmoking volunteers (mean age, 30 years; range, 22 to 52 years) with an individual mean weight of 76 kg (range, 64 to 90 kg). Within each series, the same six subjects participated in all experiments (Table 1). The time period between each experimental session for each subject was one week. The infusions were well tolerated by all subjects and no side effects were observed. All subjects were informed of the purpose of the study and possible risks involved before giving their voluntary consent to participate. The study was reviewed and approved by the institutional ethics committee.

Procedure

Study 1. Isotonic solutions of either nicotine or cotinine were infused intravenously over 1 hour at a constant rate of 3.1, 15.4, 30.9, or 61.7 nmol/min. In addition, saline solution was infused in a control study to determine baseline concentrations of nicotine and cotinine. Blood samples were collected for 5 hours: 10 minutes before and at the start of the infusion and then

every 15 minutes for the rest of the study period. Urine was collected quantitatively for 5 hours at the following times: 10 minutes before and 1, 3, and 5 hours after initiating the infusion.

Study 2. Isotonic solutions of nicotine were infused over 4 hours, at a constant rate of 1.5, 3.1, 6.2, 10.8, or 15.4 nmol/min, and cotinine solutions were infused over 4 hours at a constant rate of 10.8 nmol/min. Blood and saliva samples were collected for 8 hours, beginning at the start of the infusion—blood was taken every 30 minutes and saliva every hour. A urine sample was collected at the start of the infusion, and urine was then collected and pooled for the rest of the study period.

The solutions of nicotine and cotinine—or saline in the control studies—were infused through a catheter inserted into the forearm vein of one arm and blood was sampled through a catheter introduced into a vein of the contralateral forearm. As soon as the blood had been drawn, it was transferred to a Vacutainer tube (4.5 ml; Becton Dickinson, Plymouth) containing a citrate solution (0.5 ml; 0.105 mol/L), and a few hours later the plasma was separated and transferred into a glass tube. Saliva samples (1 ml each) were gathered during 2 minutes directly into glass tubes. The volume and pH of the urine voids were measured and 20 ml aliquots were transferred into glass tubes. The plasma, saliva, and urine samples were kept frozen (-18°C) until analyzed.

METHODS

The concentrations of nicotine and cotinine in plasma and urine were determined by the method of Curvall et al.⁵ To samples of plasma, saliva, and urine (1 ml), appropriate amounts of the internal standards were added, *N*-methylanabasine for nicotine and *N*-

Table II. Pharmacokinetic parameters of nicotine and cotinine after intravenous infusions of different doses of nicotine and cotinine during 1 hour

Parameters	Saline solution	Dose of nicotine (nmol · min ⁻¹)			
		3.1	15.4	30.8	61.7
Nicotine					
C _{max} (ng · ml ⁻¹)	—	2.0 ± 0.6	1.6 ± 0.4	3.3 ± 0.8	4.2 ± 0.7
T _{max} (hr)	—	0.6 ± 0.1	0.9 ± 0.2	0.7 ± 0.2	1.0 ± 0.1
AUC ₀₋₁ (ng · ml ⁻¹ · hr)	3.7 ± 0.6	5.9 ± 0.8	4.9 ± 0.8	8.8 ± 1.4	9.7 ± 1.3
Ae (% of dose)	—	9.9 ± 5.5	4.8 ± 3.0	4.7 ± 1.5	3.7 ± 2.8
Cotinine					
C _{max} (ng · ml ⁻¹)	—	0.7 ± 0.2	1.5 ± 0.4	3.0 ± 0.7	6.3 ± 0.9
T _{max} (hr)	—	3.4 ± 0.5	3.1 ± 0.6	2.3 ± 0.8	3.3 ± 0.4
C _{ss} (ng · ml ⁻¹)	0.2 ± 0.2	0.4 ± 0.1	1.2 ± 0.3	2.9 ± 0.5	5.5 ± 0.9
AUC ₀₋₁ (ng · ml ⁻¹ · hr)	1.1 ± 1.4	1.8 ± 0.3	5.1 ± 1.2	11.9 ± 1.9	22.3 ± 3.7
Ae (% of dose)	—	7.3 ± 2.6	3.3 ± 1.6	2.5 ± 0.8	3.2 ± 1.2
F _m	—	—	—	—	—

Data are mean values ± SD.

C_{max}, Maximal concentration; T_{max}, time to maximal concentration; C_{ss}, average plateau concentration; AUC, area under the concentration-time curve; Ae, amount excreted; F_m, fraction of nicotine metabolized to cotinine.

ethylnorcotinine for cotinine. The samples were extracted with dichloromethane under basic conditions and the organic layer was separated and evaporated. Quantitative analysis was performed on a gas chromatograph (Varian model 3700, Walnut Creek, Calif.), equipped with an all-glass capillary injector, a fused silica capillary column (SP-1000, Orion Analytica, Espo, Finland), and a thermionic specific detector (TSD, Varian). The plotting of the chromatograms and the integration of the peak heights and peak areas were carried out by a Hewlett-Packard Model 3388 A (Hewlett-Packard Company, Avondale, Pa.) or a Shimadzu Model C-R3A (Tokyo, Japan) plotter/integrator. The sensitivity of this assay was 0.1 ng of nicotine and cotinine per milliliter of body fluid, with coefficients of variation of 11.6% and 12.9% for nicotine and cotinine, respectively, at concentrations of 1 ng/ml.

Calculations

Standard statistical methods were used, using the paired *t* test when applicable. Data in the text, tables, and figures are given as mean values ± SD. The maximum plasma concentration (C_{max}) and time to C_{max} (T_{max}) were determined directly from the graphic plots. The mean plateau concentration of cotinine (C_{ss}) was calculated over 1 to 4 hours after the infusion was terminated. The area under the plasma concentration-time curve (AUC) was determined by use of the trapezoidal method. The fraction of nicotine converted to cotinine (F_m) was estimated by division of the AUC values of cotinine obtained from the nicotine infusion by those from the corresponding cotinine infusion.

RESULTS

Study 1

The mean plasma concentration-time profiles after intravenous infusions of saline solution or 3.1, 15.4, 30.9, or 61.7 nmol/min of nicotine over 1 hour are shown in Fig. 1. The average values of the pharmacokinetic parameters for nicotine and cotinine, calculated from the individual plasma concentration-time curves obtained with intravenous infusions of nicotine and cotinine, respectively, are listed in Table II.

The C_{max} values for nicotine were reached 0.6 to 1 hour after the start of the infusion and did not correlate with the dose. The AUC values rose with the dose for the three doses of 15.4, 30.9, and 61.7 nmol/min, but the increase was not proportional. Neither was a correlation found between the dose and the urinary recovery of nicotine. The C_{max}, AUC, and Ae (amount excreted) values for the lowest dose (3.1 nmol/min) were influenced by the background values of nicotine in the body and were therefore not proportional to those obtained for the higher doses.

The C_{ss} values of cotinine increased proportionally with the dose and were reached 2.3 to 3.4 hours after the initiation of the nicotine infusion. As can be seen from the plasma cotinine concentration-time curves, a clear plateau level (C_{ss}) was reached 1 to 4 hours after the infusion was terminated. A significant linear relationship existed between the nicotine infusion rate and the mean plateau concentration of cotinine, as calculated over that period of time. The mean regression line, which was forced through zero, was calculated as follows:

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Dose of cotinine (nmol · min ⁻¹)				
3.1	15.4	30.8	61.7	
1.3 ± 0.5	3.2 ± 0.7	6.1 ± 1.2	11.9 ± 1.8	
1.3 ± 0.5	1.0 ± 0.1	1.1 ± 0.2	1.2 ± 0.3	
0.8 ± 0.3	2.3 ± 0.3	4.4 ± 1.2	8.1 ± 0.6	
4.3 ± 1.5	11.8 ± 1.6	22.1 ± 5.3	40.5 ± 4.1	
10.1 ± 3.8	6.3 ± 1.9	6.5 ± 2.2	5.8 ± 1.3	
0.42	0.43	0.54	0.55	

Cotinine concentration (ng/ml) =
0.53 × Nicotine infusion rate (μg/min)

($r = 0.97$, $p < 0.001$). The slopes of the individual regression lines ranged from 0.43 to 0.64, and the lowest individual correlation coefficient was 0.98. There was also a significant relationship between the AUC for the plasma concentration-time curves and the nicotine dose ($r = 0.99$). About the same amount of cotinine as nicotine was excreted in the urine and it ranged from 2.5% to 3.3% of the dose, except for the lowest dose, which was affected by background values of nicotine.

The C_{max} values after the 1-hour cotinine infusion increased in proportion to the infusion rate and were reached 1 to 1.3 hours after the beginning of the infusion. Also the C_{ss} and AUC values were significantly correlated with the cotinine infusion rate. The fraction of nicotine that was metabolized to cotinine (F_m) ranged from 0.42 to 0.55. Cotinine is partly excreted unchanged in urine and, in keeping with the results based on plasma concentrations, the percentage of urinary recovery was independent of the dose.

Study 2

The mean plasma cotinine concentration-time curves after the 4-hour intravenous infusions of saline solution and 1.5, 3.1, 6.2, 10.8, or 15.4 nmol/min of nicotine are shown in Fig. 2. The pharmacokinetic parameters for nicotine and cotinine (C_{ss} , AUC, and F_m), as calculated from the plasma and saliva concentration-time data, and the amount of nicotine and/or cotinine excreted in the urine, are listed in Table III.

The plasma nicotine concentration rose during the infusion, but no clear C_{max} values could be obtained

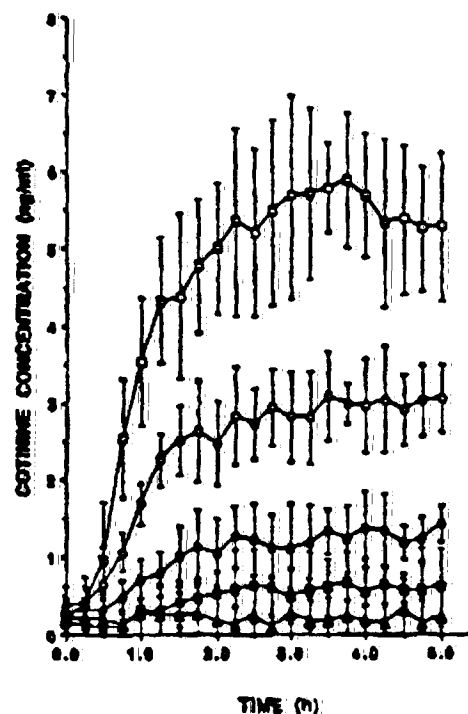


Fig. 1. Study 1. Mean plasma cotinine concentration-time curves obtained during and 4 hours after 1 hour of intravenous infusions of saline solution (triangles), and nicotine at four different rates: 3.1 nmol/min (closed squares), 15.4 nmol/min (closed circles), 30.9 nmol/min (open circles), and 61.7 nmol/min (open squares).

from the nicotine concentration-time curves. The saliva nicotine concentration, however, increased markedly during the infusion and reached C_{max} 3 to 4 hours after initiation of the infusion. The C_{max} values, in order of increasing dose, were 3.7, 6.0, 11.5, 16.8, and 21.0 ng/ml. Both the plasma and saliva nicotine AUC values increased with the dose, but the increments were not directly proportional to the dose. The fraction of nicotine excreted unchanged in the urine was 1.7% to 3.2% of the dose, except for the lowest dose, for which the share was higher (6.6%) because of the background concentration of nicotine.

A distinct plateau level of plasma cotinine was observed from 1 to 4 hours after termination of the infusion. A linear relationship existed between this plateau level and the nicotine dose. When forced through zero, the regression line was as follows:

Cotinine concentration (ng/ml) =
1.75 × Nicotine infusion rate (μg/min)

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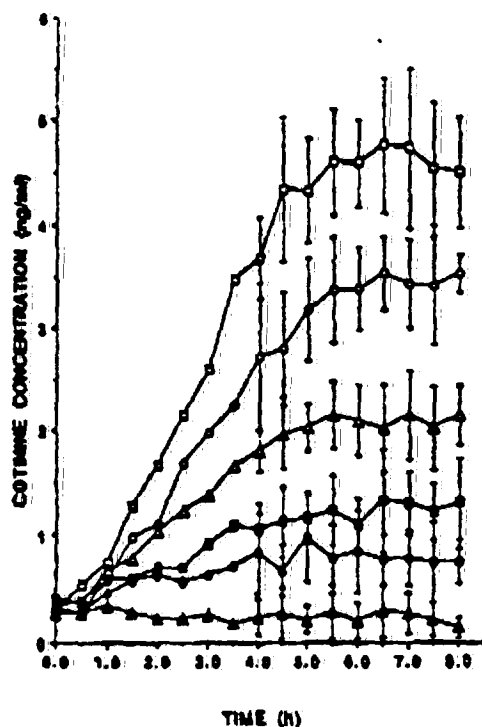


Fig. 2. Study 2. Mean plasma cotinine concentration-time curves obtained during and 4 hours after 4-hour intravenous infusions of saline solution (closed triangles) and nicotine at five different rates; 1.5 nmol/min (closed circles), 3.1 nmol/min (closed squares), 6.2 nmol/min (open triangles), 10.8 nmol/min (open circles) and 15.4 nmol/min (open squares).

($r = 0.98$, $p < 0.001$). The slopes of the individual regression lines were within the range 1.56 to 1.97, and the smallest correlation coefficient was 0.98. The saliva cotinine concentration-time curves also showed clear plateau levels from 1 to 4 hours after the infusion. The regression line for the relationship between the nicotine dose and the saliva plateau concentration of cotinine was calculated as follows:

$$\text{Cotinine concentration (ng/ml)} = 2.26 \times \text{Nicotine infusion rate } (\mu\text{g/min})$$

($r = 0.97$, $p < 0.001$). The slopes of the individual regression lines ranged from 1.82 to 2.84, the smallest correlation coefficient being 0.96. The correlation between the salivary and plasma cotinine concentrations of samples collected 1 to 4 hours after the infusion is shown in Fig. 3. As can be seen from the slopes of the

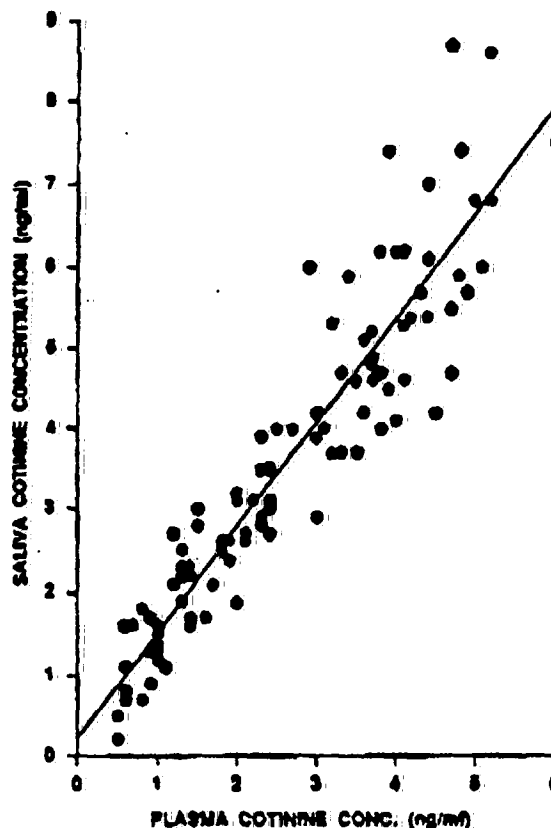


Fig. 3. Study 2. Relationship between saliva and plasma steady-state concentrations of cotinine after intravenous infusions of five different doses of nicotine over 4 hours ($y = 1.27x + 0.24$; $r = 0.93$).

regression line, the ratio between salivary and plasma cotinine is 1.27. The plasma cotinine AUC values were significantly correlated with the dose ($r = 0.97$). The quotients of the saliva and plasma AUC values were 1.39, 1.39, and 1.37 for the three doses 6.2, 10.8, and 15.4 nmol/min. The saliva AUC values for the lowest doses were excluded because of an insufficient number of samples. The amount of cotinine excreted ranged from 2.2% to 3.6% of the nicotine dose, except for the lowest dose.

Both the plasma and the saliva cotinine concentrations increased during the infusion of 10.8 nmol/min of cotinine over 4 hours; the average plasma C_{max} value (5.4 ng/ml) and the average saliva C_{max} value (7.3 ng/ml) were reached 4.5 and 4.0 hours, respectively, after initiation of the infusion. The saliva to plasma cotinine AUC ratio was 1.30, which is in good agree-

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Parameters	Saline solution	Dose of nicotine (nmol · min ⁻¹)					Dose of cotinine (nmol · min ⁻¹)
		1.5	3.1	6.2	10.8	15.4	10.8
Nicotine							
Plasma AUC ₀₋₄ (ng · ml ⁻¹ · hr)	4.5 ± 0.9	4.9 ± 0.3	5.8 ± 1.3	7.1 ± 1.7	10.3 ± 1.3	11.6 ± 2.8	
Saliva AUC ₀₋₄ (ng · ml ⁻¹ · hr)		17.4 ± 3.4	33.2 ± 15.3	42.7 ± 10.7	66.1 ± 26.5	95.8 ± 10.2	
Ae (% of dose)		6.6 ± 4.2	3.2 ± 1.2	1.7 ± 0.8	2.6 ± 1.8	3.1 ± 3.0	
Cotinine							
Plasma C _{ss} (ng · ml ⁻¹)	0.2 ± 0.2	0.6 ± 0.2	1.2 ± 0.3	2.1 ± 0.3	3.4 ± 0.3	4.6 ± 0.5	4.3 ± 0.6
Saliva C _{ss} (ng · ml ⁻¹)	0.3 ± 0.1	1.0 ± 0.4	1.8 ± 0.6	2.9 ± 0.4	4.4 ± 0.5	6.2 ± 0.6	5.1 ± 0.8
Plasma AUC ₀₋₄ (ng · ml ⁻¹ · hr)	1.4 ± 1.1	3.7 ± 1.0	6.9 ± 1.5	12.4 ± 1.6	18.3 ± 2.8	25.2 ± 3.0	29.4 ± 3.0
Saliva AUC ₀₋₄ (ng · ml ⁻¹ · hr)				17.3 ± 3.1	25.5 ± 3.3	34.6 ± 5.1	38.3 ± 3.0
Ae (% of dose)		6.1 ± 1.4	3.6 ± 1.6	3.6 ± 0.8	2.1 ± 0.5	2.5 ± 0.6	3.8 ± 1.5
F _m							0.62

Data are mean values \pm SE.

C_{ss} , Average plateau concentration; AUC, area under the concentration-time curve; As, amount excreted; F_m , fraction of nicotine metabolized to cotinine.

ment with the results from the corresponding infusion of nicotine. Only 4% of the cotinine dose was excreted unchanged in the urine during the infusion and the following 4 hours. The fraction of nicotine metabolized to cotinine (F_m) was 0.62.

DISCUSSION

Nicotine and cotinine concentrations in body fluids have been widely used to estimate the intake of nicotine during smoking or exposure to environmental tobacco smoke. Because nicotine has a short half-life in body fluids (2 hours),² its concentration in blood is sensitive to the time of blood sampling. Cotinine, however, has an elimination half-life of about 15 hours⁴ and is therefore more useful as a marker of nicotine intake. However, the estimation of nicotine intake from cotinine concentrations in body fluids is valid only if the metabolism of nicotine and the subsequent elimination of cotinine are independent of the dose. The pharmacokinetics of nicotine and cotinine have been evaluated in smokers and nonsmokers at concentrations usually achieved by smokers, and little is known about the kinetics of these compounds at concentrations found in nonsmokers exposed to environmental tobacco smoke nicotine. Galeazzi et al.⁶ documented a linear relationship between the nicotine intake and the steady-state

plasma concentrations of cotinine when nicotine was given intravenously at intervals over 4 days to simulate the intake by smokers. The suitability of cotinine as a marker of environmental tobacco smoke nicotine exposure has only been evaluated in field studies; no data are available on the relationship between low-dose nicotine intake and cotinine concentrations in nonsmokers.

In the present investigation, nicotine was infused over 1 and 4 hours, respectively, to simulate the nicotine intake of passive smokers. The nicotine infusion rates were designed to simulate the absorption rates of nicotine during environmental tobacco smoke exposure and the resulting cotinine concentrations were similar to or higher than those obtained by nonsmokers in indoor public places.⁹ Studies of nicotine and cotinine kinetics in body fluids of nonsmokers require access to rapid and sensitive analytic methods. As can be seen in Figs. 1 and 2, which show the cotinine concentrations obtained after infusions of different doses of nicotine over 1 and 4 hours, respectively, the interindividual differences in the pharmacokinetics of cotinine were small in this group of nonsmokers. The linear relationship between the AUC values of cotinine and the different infusion doses of nicotine and cotinine show that both the metabolism of nicotine to cotinine and the elimination of cotinine are dose-independent. These

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findings are in good agreement with results obtained after administration of cotinine to nonsmokers at doses usually achieved by smokers.^{9,9} The fraction of nicotine that was metabolized to cotinine was about 0.5, which is somewhat lower than the fraction previously reported for smokers.¹⁰ This discrepancy is caused either by differences in the metabolism of nicotine to cotinine between smokers and nonsmokers or by differences in the experimental setup.

A clear plateau level of cotinine was obtained from 1 up to 4 hours after termination of the infusion. In accordance with the dose-independent kinetics of cotinine, the amount of nicotine infused over 1 and 4 hours was directly proportional to the average plateau concentration of cotinine. The regression line:

$$\text{Cotinine concentration (ng/ml)} = 0.5 \times \\ \text{Nicotine infusion rate } (\mu\text{g/min}) \times \text{absorption time (hr)}$$

can be used as a standard curve for estimating the average nicotine intake at environmental tobacco smoke exposure levels over periods 1 up to 4 hours. Environmental tobacco smoke exposure studies of large populations in real-life situations require invasive sampling methods. In study 2, both plasma and unstimulated saliva samples were collected and it was found that the salivary cotinine concentration also reached a plateau level 1 to 4 hours after completion of the infusion. The nicotine dose correlated significantly both with the salivary cotinine AUC values and with the cotinine concentrations at steady state. A significant relationship was also observed between the salivary and plasma cotinine concentrations ($r = 0.97$), the regression coefficient being 1.27, which is in accordance with findings obtained for smokers.¹¹ It can thus be concluded that salivary cotinine is a good biochemical marker for nicotine and provides the same information on nicotine intake as does plasma cotinine.

The urinary cotinine concentrations obtained after the 1-hour infusion of nicotine did not reach a plateau level within 5 hours. Individual urinary excretions of cotinine did not correlate with the dose, but the average cotinine concentrations obtained 2 and 4 hours after completion of the 1-hour infusion did and so did the average total amount excreted during and after the infusion. Also the average total amount excreted during and after the 4-hour infusion correlated with the dose. Accordingly, because of interindividual differences in cotinine excretion, estimation of nicotine intake after environmental tobacco smoke exposure has to include several subjects and the total urine volume up to 4 hours after the nicotine intake has to be collected in a group of subjects.

In conclusion, these studies show that the metabolism of nicotine to cotinine and the elimination of cotinine are not dose-dependent at the low doses of nicotine to which nonsmokers are exposed by environmental tobacco smoke. Both plasma and salivary cotinine levels are useful individual biochemical markers for nicotine intake at doses similar to those obtained in indoor public places. Individual urinary cotinine concentrations, however, could not be used for the estimation of nicotine intake in nonsmokers. Moreover, the use of urinary cotinine requires exact sampling times or collection of the whole urine void. Plateau concentrations of plasma and salivary cotinine were reached 1 to 4 hours after the administration of nicotine and were found to be directly proportional to the nicotine infused over 1 to 4 hours. By use of the equation:

$$\text{Cotinine concentration (ng/ml)} = 0.5 (\times 1.3) \times \\ \text{Nicotine infusion rate } (\mu\text{g/min}) \times \text{absorption time (hr)}$$

the average intake of nicotine during environmental tobacco smoke exposure could be calculated from a single plasma (saliva) cotinine determination.

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A Comparison of Plasma and Urinary Nicotine and Cotinine Levels in Smokers and Nonsmokers: Nicotine Excretion Pathways Are Possibly Differential According to the Dosage of Tobacco Smoke Uptake

S. Itani, E. Higashi, and Y. Shimizu

Summary

Nicotine and cotinine levels in plasma (pnic, pcot) and urine (unic, ucot) sampled at a steady state were studied in 83 smokers and 90 nonsmokers.

Although there was considerable interindividual variability in measured levels for any given self-reported number of cigarettes smoked per day (CPD), some regularity was discovered among these four levels in relation to CPD.

Average pnic and pcot increased in proportion to CPD up to 15 CPD and more than 50 CPD, but from 20 to 40 CPD, a discrepancy involving more pcot and less pnic was discovered. Average unic rose more rapidly than average ucot up to 15 CPD, but at above 20 CPD, ucot increased more predominantly than unic. In nonsmokers, these four levels were, if detectable at all, extremely low, particularly as concerns cotinine.

The results indicate that the nicotine excretion mechanism may be differential according to the recent uptaken nicotine dosage. In most nonsmokers, the main pathway for nicotine excretion is the nicotine to nicotine route (NNR). The nicotine to cotinine route (NCR) may act as a backup. Light smokers may acquire the ability to convert a greater amount of nicotine to cotinine in proportion to CPD, with both pathways equally available.

Predominant production and excretion of cotinine is suggested for smokers who smoke more than 20 CPD, with the main route replaced by NCR. In extremely heavy smokers who smoke more than 50 CPD, it is suggested that the transaction limits of the nicotine to cotinine conversion system are exceeded and that both pathways are at maximum availability.

As concerns the indicator of ETS exposure for nonsmokers, all four levels are not always completely measurable. It is suggested that pnic is the most sensitive, but all four markers are equally necessary to estimate the low dosage of tobacco smoke uptake.

Introduction

Our previous statistical comparison of the levels of plasma nicotine (pnic), cotinine (pcot) and thiocyanate, urinary creatinine ratios of nicotine (unic), cotinine (ucot) and thiocyanate as well as COHb and expired carbon monoxide in a cross-sectional study revealed that these tobacco smoke uptake parameters (TSUPs) were more significantly elevated in the smokers than in the nonsmokers. The results suggested that pcot and ucot are the most suitable parameters for discrimination of smokers from nonsmokers [15].

Recently, urinary cotinine determination has frequently been used as an excellent TSUP in the study of ETS effects. Significantly elevated ucot levels have been pointed out for nonsmokers living with smokers as compared with those for nonsmokers living with

nonsmokers, particularly when determination is made with the radioimmuno assay (RIA) method [14, 20]. In our study population, however, only 2% of the 90 nonsmokers showed detectable amount of ucot by gas chromatographic (GC) method. This is extremely low in frequency compared with the previous reports.

In order to increase the sensitivity and accuracy of the measurement of ETS effect, we have undertaken to reevaluate the determined levels of nicotine related parameters (i.e., pnic, unic, pcot and ucot) in smokers and nonsmokers. Nicotine and its major metabolite-cotinine are considered to be derived from tobacco only [4]. The determined levels of these markers in blood and urine on the same occasion may reflect a dynamic state of nicotine metabolism within the body at the time.

Accordingly, possible nicotine excretion pathways as well as the significance of in vivo nicotine to cotinine conversion in relation to daily cigarette consumption are also discussed.

Materials and Methods

Subjects

Eighty-three smokers aged 23 to 61 and 90 healthy nonsmokers aged 27 to 55 in natural condition were considered in the study. Most were office workers leading ordinary social lives. No particular attention was paid to the grade of ETS exposure to nonsmokers.

Analytical Methods

In most cases, random spot blood and urine samples were taken at almost the same time in the early afternoon. Smoking was prohibited for three hours before sampling. These plasma and urine samples (only cases with acidic urine samples were made available in the study) were stored at -20°C until analysis.

Nicotine and cotinine levels were determined by a modified FTD-GC method as previously described [8, 9, 15]. Urinary levels of these components were expressed by creatinine ratios.

Evaluation of the Four Parameters in Relation to the Number of Cigarettes Consumed Per Day

Eighty-three smokers were divided into seven groups according to the self-reported number of cigarettes smoked per day (CPD) as follows: 1 to 9 (5 CPD), 10 (10 CPD), 11 to 19 (15 CPD), 20 (20 CPD), 30 (30 CPD), 40 (40 CPD) and more than 50 CPD (50 CPD).

In order to estimate the relationship of the four parameters to CPD, the four individual levels were compared in a graphic pattern analysis. The four levels determined for each subject were plotted on the respective axes of XY orthogonal co-ordinates and connected with four lines to form a tetragon-figure, i.e., individual components on the upper, left, right and lower axes represent the levels determined for pnic, unic, pcot and ucot, respectively. Pattern analysis was performed taking notice of size and deviation in the form of the tetragon. Prominent deviations in the upward, left, right and downward direction are expressed by the letters N, n, C and c, respectively. For nonsmokers, all the

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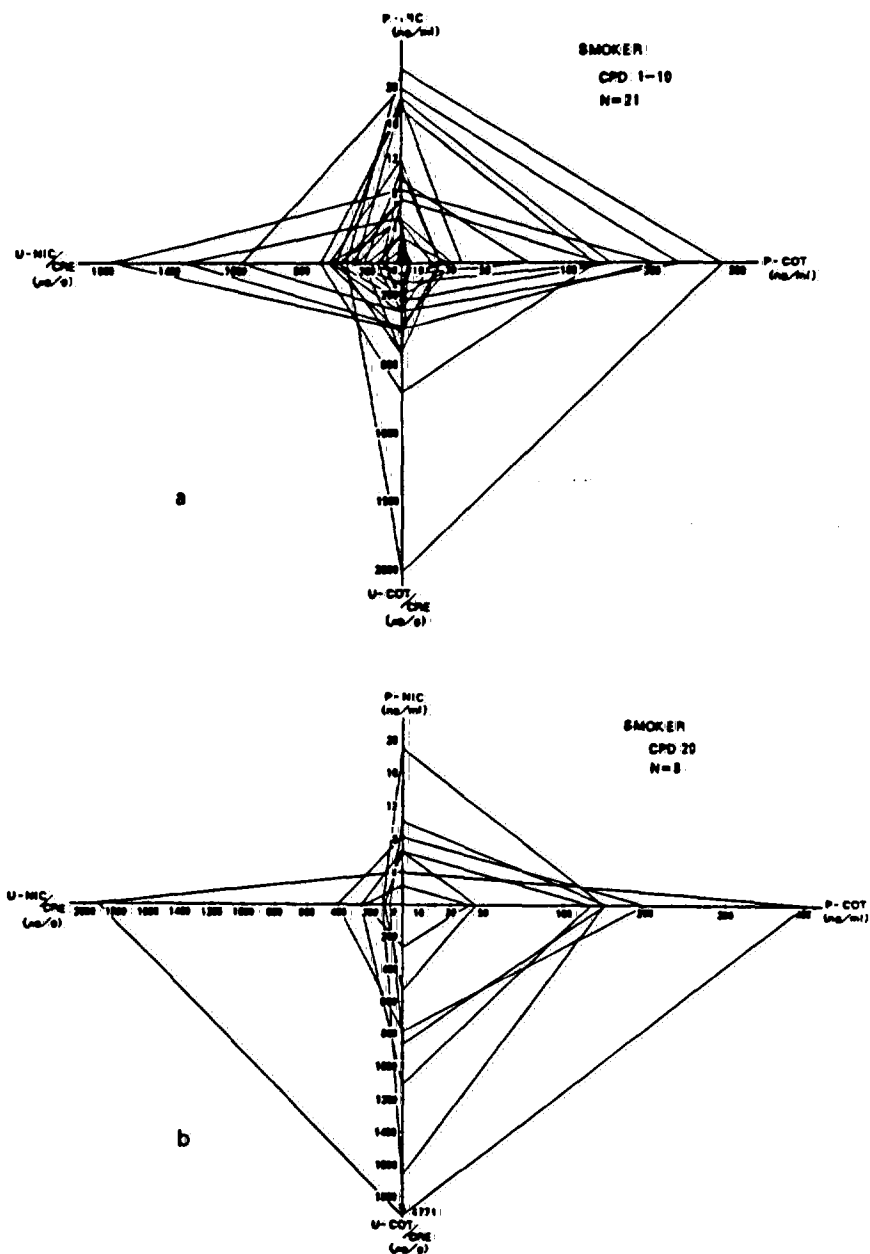


Fig. 1 a, b. Individual pnic, unic, pcot and ucot levels in (a) 21 light smokers (≤ 10 CPD) and in (b) eight 20 CPD smokers. Notice the tendency toward more pnic with less ucot in the former and, in contrast, toward less pnic and less unic with more ucot in the latter.

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detectable components, if any, are described by the corresponding primed letters N', n', C' and c', and no detectable one is expressed by the letter o.

Average nicotine to cotinine ratios in plasma and urine were also compared among smoker-groups in order to detect whether or not a tendency exists in comparative changes of nicotine and cotinine levels.

Results

Graphs of individual levels of all four markers of (a) 21 light smokers (≤ 10 CPD) and (b) eight heavy smokers ($= 20$ CPD) are shown in Fig. 1 a, b, respectively.

It is clear that there was a high variability among the levels determined for different individuals who smoked almost the same number of cigarettes even for light smokers. However, marked differences between these two figures are as follows: Light smokers (a) showed a tendency to show higher levels of pnuc with lower levels in ucot. Heavy smokers (b), by contrast, generally showed a tendency to have lower levels of pnuc and unuc with higher levels of ucot. Thus, most of them could be classified into several patterns based on the size and deviated form of the tetragon using the letters defined above.

Distribution ranges, averages and results of pattern analysis of the levels of the four markers determined in individual smoker-groups and in nonsmokers are summarized in Table 1.

Characteristics of Tetrasons Formed by the Four Average Levels in Individual Smoker-groups

The four average levels in each smoker-group are shown in Fig. 2. The area between the axes of pnuc and unuc is considered as the nicotine-related plane, while the area between the axes of pcot and ucot is defined as the cotinine-related plane.

The smallest tetragon labeled 5 CPD in the nicotine-related plane is that for 5 CPD. It indicates higher pnuc and unuc levels but lower pcot and ucot levels. The 10 CPD tetragon shows a high level of pnuc and a low level of ucot. This tendency is found more markedly in the 15 CPD tetragon. Even higher levels of pnuc and unuc as well as of pcot are noticed, but the level of ucot remains low. The 20 CPD tetragon, labeled in the cotinine-related plane, is quite different from the previous tetrasons. This tetragon is characterized by considerably lower levels of pnuc and unuc with predominantly higher level of ucot compared with the 10 and 15 CPD tetrasons, as shown in Fig. 1 (b).

One of the characteristics of the 30 and 40 CPD tetrasons which follow 20 CPD tetragon appears to be another increase in unuc level in addition to that of the 20 CPD tetragon. The pnuc level remains relatively low, however. The 50 CPD tetragon is also quite different, characterized by extremely high levels of all the components.

Relationship of Average Plasma and Urinary Nicotine and Cotinine Levels to Daily Cigarette Consumption

As shown in Fig. 3 (a), pcot levels seem to have a roughly positive correlation to CPD.

Contrary to our expectations, however, pnuc levels appear to have no positive correlation to CPD. The level of pnuc was observed to peak first at 15 CPD, then fall

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Table 1. Distribution ranges, averages and patterns of plasma and urinary nicotine and cotinine levels in smoker-groups by CPD and in nonsmoker-group

Group (CPD)	No. of subjects	Range (Average)				Patterns
		pnic ng/ml	unic µg/g	pcot ng/ml	ucot µg/g	
5	6	0- 7.4 (3.0)	0-1,280 (254.5)	0- 31 (7.0)	0- 375 (144.5)	o* (2), N (1), nC (3)
10	15	3.4-20.0 (11.1)	102-1,784 (422.4)	6-234 (93.3)	134- 771 (428.7)	N (1), n (4), Nn (1) NC (1), Nc (1), nC (2) nc (1), NnC (3), NnCc (1)
15	8	4.0-22.4 (15.0)	71-4,128 (961.3)	37-392 (208.0)	140-1,068 (513.1)	n (1), Nn (1), Nc (3) Cc (1), NnC (1), Nnc (1)
20	8	2.3-19.0 (8.6)	108-1,917 (409.0)	42-392 (190.0)	266-4,771 (1,459.0)	n (1), c (1), Cc (2) NCc (2), nCc (2)
30	11	2.5-21.4 (9.6)	90-1,860 (843.6)	111-720 (235.9)	818-2,300 (1,572.1)	N (2), Nc (1), Nnc (1) nC (3), Cc (1), c (2) NnCc (1)
40	23	0.6-21.4 (6.4)	49-3,600 (872.6)	65-635 (239.2)	228-3,617 (1,569.2)	Nn (1), NCc (2), nc (3) nCc (3), Cc (1), c (11) NnCc (2)
50 ≤	12	5.9-37.0 (21.1)	137-5,603 (1,866.0)	310-557 (375.0)	1,145-5,182 (2,812.2)	NCc (2), nCc (1), NnCc (9)
Nonsmokers	90	0- 8.6	0- 120	0- 6	0- 28	o* (75), N' (4), n' (1) N'n' (2), N'C' (6), N'n'C'c' (2)

* No measurable amount discovered.

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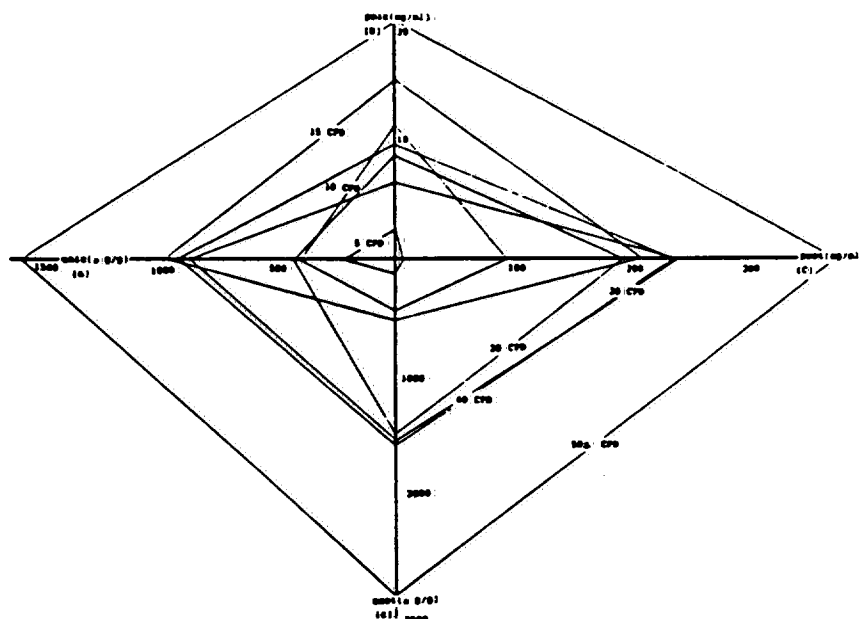


Fig. 2. Graph of average pnic, unic, pcot and ucot levels in smoker-groups divided by CPD. Compare the characteristic size and deviated form of each tetragon among inter-smoker-groups

rapidly at 20 CPD. This was followed by a gradual decrease up to 40 CPD, then a rapid increase at 50 CPD.

Pnic to pcot ratios in individual smoker-groups up to 40 CPD indicate a somewhat inverse correlation to CPD. In addition, significant differences in pnic/pcot ratios were found between 10 and 20 ($p < 0.05$); 10 and 30 ($p < 0.05$); and 10 and 40 CPD ($p < 0.001$), respectively. These results indicate that pnic and pcot levels increase in proportion to the daily cigarette consumption up to 15 CPD and more than 50 CPD, but in the intermediate region from 20 to 40 CPD, a discrepancy of more cotinine but less nicotine in plasma is found.

These relations in urinary components are shown in Fig. 3 (b). The ucot levels increased in proportion to the increase in CPD, but rapid stepped rises were discovered at 20 and 50 CPD. As for unic levels, an initial peak and trough were found at 15 and 20 CPD, respectively. Unlike pnic levels, however, unic levels increased gradually from there up to 40 CPD. This was followed by a final marked increase at 50 CPD.

Unic to ucot ratios in individual smoker-groups were somewhat discrepant from those in plasma. An initial marked peak and trough were observed at 15 and 20 CPD, respectively. Then a gradual increase occurred. Significant differences in unic/ucot ratios were found between 10 and 20 ($p < 0.01$); 10 and 30 ($p < 0.05$); 10 and 40 ($p < 0.001$); and 15 and 20 CPD ($p < 0.01$), respectively.

These results indicate that nicotine levels in urine rise more rapidly than cotinine levels up to 15 CPD, but that at above 20 CPD, the increase in cotinine levels is more predominant than that in nicotine levels.

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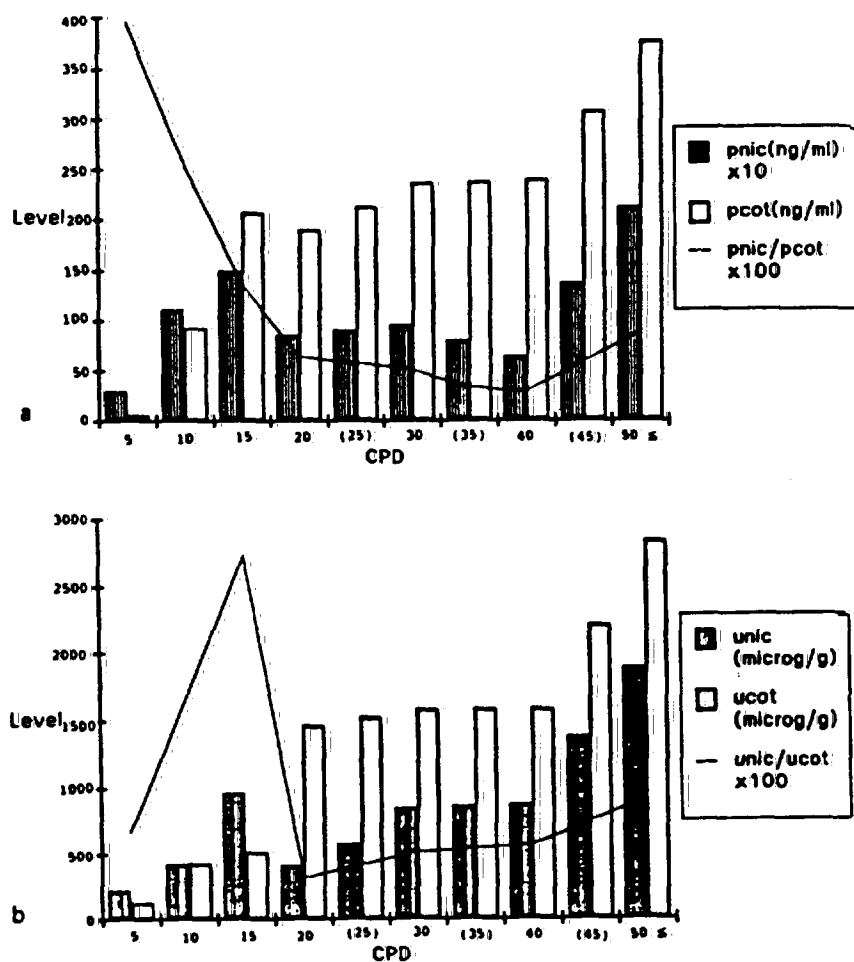


Fig. 3a, b. Relationship of (a) average pnic, pcot and pnic/pcot levels to CPD and of (b) unic, ucot and unic/ucot levels to CPD. Individual levels indicated by the number in parentheses indicate the estimated values for the corresponding CPD

Plasma and Urinary Nicotine and Cotinine Levels in Nonsmokers (Table 1, Fig. 4)

Out of 90 nonsmokers, at least one of the four parameters could be detected positively in 25 cases (28%). Unlike those for smokers, the amounts were extremely low for nonsmokers with detectable levels of these parameters, particularly as concerns cotinine levels, and all four levels were not always completely measurable. Detectable ucot levels could be measured in only two cases (2.3%).

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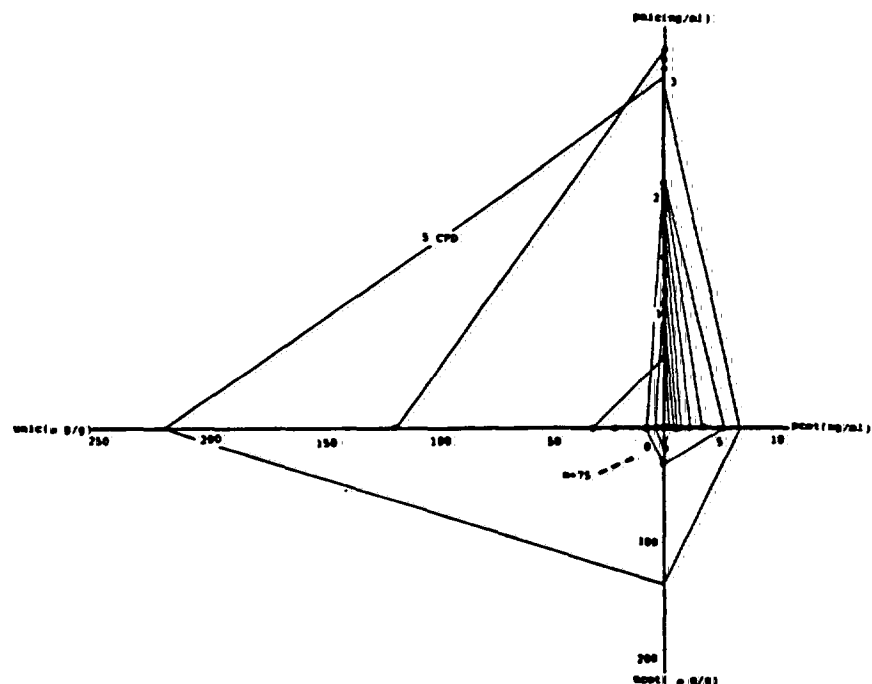


Fig. 4. Graph of pnic, unic, pcot and ucot levels in nonsmokers ($n = 90$). The outer tetragon represents the averages of these levels in 5 CPD smokers as shown in Fig. 2

Discussion

The nicotine excretion pathways following cigarette smoke inhalation may be considered to be as follows. A large portion of nicotine absorbed will finally be excreted into the urine in unchanged form through the nicotine to nicotine route (NNR) or is excreted in a converted form such as cotinine through the nicotine to cotinine route (NCR) [20]. Cotinine, the primary metabolite of nicotine, is formed in the liver in a two-step process [7, 17]. It has been recognized that at a steady state, as in the early afternoon, the rate of metabolite excretion reflects the rate at which the metabolites are generated [4].

Our findings (Fig. 5) that these four levels increased in proportion to the increase in CPD up to 15 CPD and that unic level rose more rapidly than ucot level indicate that NNR is the main route but that NCR is also made available in light smokers (1 ~ 15 CPD). Similarly, in smokers who smoked 20 to 40 CPD, the findings indicate that the main route is replaced by NCR. In smokers who smoked more than 50 CPD, both pathways are suggested to be fully used in order to excrete the rather large amount of uptaken nicotine.

Our findings that cotinine levels were extremely low or absent as compared with nicotine levels in the nonsmokers in whom any of these markers could be detected indicate that NNR is the main route in nonsmokers. This may be supported by an early finding of Beckett and Triggs [1] who observed that the excretion of nicotine was greater in nonsmokers than in smokers.

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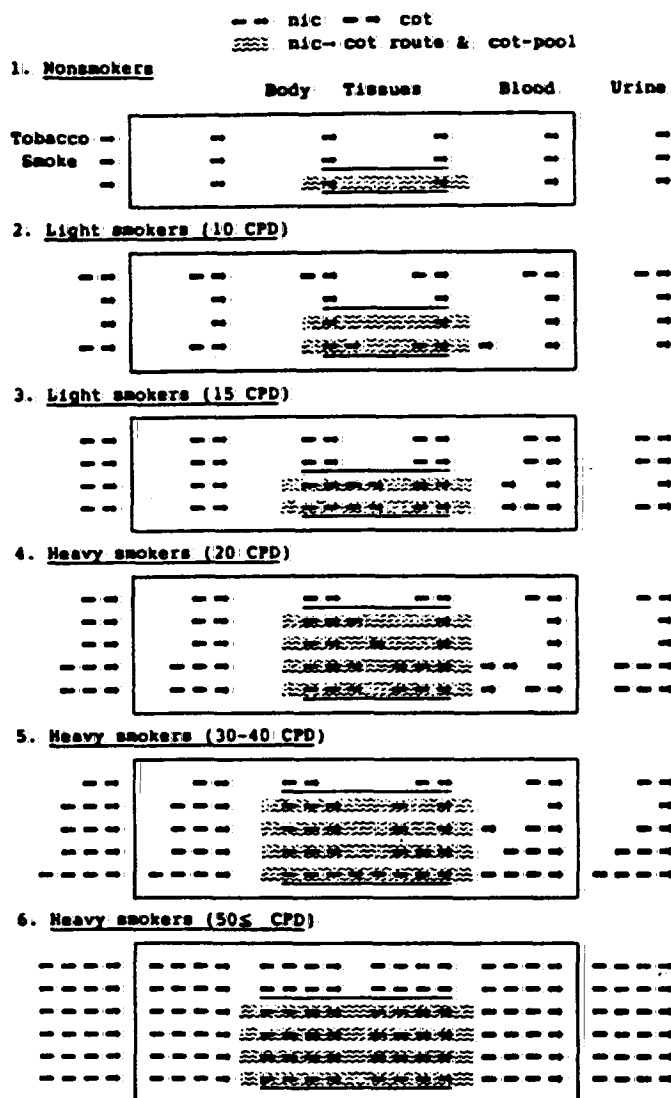


Fig. 5. A tentative schema of nicotine to nicotine (NNR) and nicotine to cotinine (NCR) pathways in relation to CPD based on the present findings. In most nonsmokers, NNR and NCR may be the main and salvage pathways, respectively. Switching of the main pathway from NNR to NCR may be differential in most smokers according to the usual nicotine dosage from tobacco smoke uptake

A time course study on the rise and decline of these four levels after smoking a single cigarette by current nonsmokers is in progress. The preliminary findings show an initial plasma nicotine peak not so inferior to the smoker's peak level at 5 min and of a sudden fall at 15 min followed by a gradual decline until 48 h and a rapid predominant increase in

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urinary nicotine level from immediately after smoking to 60 min with a transient appearance of cotinine in plasma and urine. This also supports the theory that NNR is the main route in nonsmokers.

It has been discovered that chronic nicotine administration to rats or mice does not increase the rate of nicotine metabolism [6], but that among humans, the nicotine metabolism is faster in smokers than in nonsmokers [2, 12, 18, 19]. This evidence may be in agreement with our present findings. The induction of hepatic microsomal enzymes as explanation, however, is equivocal since reports of inhibition as well as induction have been made occurred [2, 12, 18].

Our present explanation of the switching from the main pathway from NNR to NCR as a result of an increase in CPD may be coincident with these literature findings for the following reasons.

1. Most smokers, who may regulate nicotine intake by modifying puff and inhalation patterns [5], may acquire an ability to convert a greater amount of nicotine to cotinine rapidly, possibly due to the induction in increasing activity of cytochrome P 450 in the liver [7, 16, 17, 18] in proportion to CPD up to about 40 CPD. The levels of the markers in most of the 50 \geq CPD smokers suggest that this enzyme system has a limited capability, and is not able to treat excess nicotine in a short duration. Rapid cotinine formation in smokers may be a defence mechanism in a sense, since cotinine is much less toxic than nicotine in rats [13], and probably in humans as well. High plasma nicotine with the inhibition of cotinine formation as seen in most 50 CPD smokers may be a sign of "true" nicotine toxicity.
2. Interindividual variability at any given CPD as shown in Fig. 1 may result partly from individual differences in enzyme activity. The conversion rate and amount of nicotine to cotinine is suggested to be highly dependent, however, on the recent uptaken quantity of tobacco smoke.

Out of 90 nonsmokers in a natural condition, positive levels of at least one parameter were found out in 28%. The pnuc level seems to be the most sensitive indicator of exposure to other people's smoke, and ucot may be a rather insensitive marker in determination of such low levels of the alkaloid as found in nonsmokers.

These findings, however, seem to be controversial to recent literature in which greater ucot levels in many more nonsmokers have been reported [10, 11, 14, 20], although the study populations and methods employed (RIA) were different from ours.

This discrepancy may partly be due to difference in detection methods. The recent international interlaboratory study on nicotine and cotinine determination has revealed that GC derived values showed reasonable agreement but that cotinine determinations in urine by RIA are less precise than those in serum. It also showed that RIA values were higher than GC values in urine samples and that relative variability is extremely high for both of GC and RIA in samples from nonsmokers [3].

As for the indicator of ETS uptake in nonsmokers, all four levels are not always completely measurable. This may be one of the characteristics of ETS effect on nonsmokers, probably a result of the use of NNR as the main pathway. This indicates that not only cotinine levels in blood and urine but also nicotine levels in blood and urine are equally important in dosage estimates for tobacco smoke uptake.

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Commentary

TITRATING EXPOSURE TO TOBACCO SMOKE USING COTININE—A MINEFIELD OF MISUNDERSTANDINGS

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(Received 12 July 1989)

DEFINITION OF THE PROBLEM

Whilst epidemiologists disagree about many things, there seems to be universal approval for the notion that objective measurement of tobacco smoke exposure is considerably more desirable than more self-reporting of smoking habits. As with all dogmata, caveats abound, one of the most practical of which is that without sacrificing reliability, the field test should be both simple and cheap. For reliability to be conserved, the ideal test would distinguish between true non-smokers, non-smokers exposed to environmental tobacco smoke (passive smokers) and smokers; maybe even between different smoking habits and consumption rates. Thus the test would have absolute sensitivity and specificity. It has become quite clear over the last two decades that, as various candidate tests have been introduced, the panacea would not be found. Pretenders to the crown, such as expired carbon monoxide, percent carboxy-hemoglobin and thiocyanate concentration in various body fluids, have failed to accede because of the ambiguities of incomplete sensitivity and specificity. Contemporary laboratory technology has answered the clarion call by developing such methodology as capillary gas chromatography-mass spectrometry of 4-aminobiphenyl-hemoglobin adducts [1], gas chromatography-thermal energy analyzer detection of urinary *N*-nitrosamino acids [2] or ³²P-postlabeling tests for smoking-related DNA adducts [3]. Whilst such analyses embody contemporary methodology coupled with great

precision, by their very nature, they fall outside the scope of typical clinical epidemiologic survey which simply wishes to relate a measure of tobacco smoke exposure to health issues. It seems that rather by default, salivary and urinary cotinine determinations have evolved as the current touchstones of tobacco exposure. Apart from its simple determination, and the availability of a rapid result at low cost, there is the added appeal that cotinine, as a relatively long-lived metabolite of nicotine, can be detected in the aforesaid fluids long after the "culprit" nicotine has dwindled to meagre levels.

Because the greatest body of experience to date with cotinine surrounds epidemiologic enthusiasts, often with scanty training in drug metabolism and pharmacokinetics, the two disciplines which underpin the basis of the use of cotinine in this context, I believe that a critical review of the premises and procedures is required in an attempt to examine the misconceptions which can lead to overinterpretation in what is otherwise an exciting new field of biochemical epidemiology.

My own dissatisfaction with indiscriminate use of cotinine as a dosimeter of tobacco smoke arises from the trade-off of knowledge for convenience. Nicotine is a pyridine alkaloid, one of at least 10 identified in cigarette smoke [4] and both the qualitative and quantitative distribution of this class of alkaloids amongst the flora, their metabolic interconversions in man, the issue of the concentration of cotinine in the saliva at the expense of plasma, together with

the extent of intersubject variability in human disposition of nicotine and its metabolites is both nebulous and poorly-understood. The complex of dynamic interactions which leads to a certain salivary or urinary concentration of cotinine at one point in time following exposure to a defined amount of airborne nicotine needs to be dissected. The purpose of this commentary is to demonstrate by such a dissection that single point cotinine concentrations can give no more than a clue to a past exposure to pyridine alkaloids of unknown amount, at an unspecified time, by an unknown route of entry and from unknown origins.

SOURCES OF NICOTINE AND COTININE

One of the principal premises of the practice of cotinine biomonitoring is that nicotine, and hence cotinine, is a tobacco-specific alkaloid. Cotinine is variously described as "a particularly specific and sensitive marker of exposure to tobacco smoke" [5], "a useful and reliable indicator of nicotine intake" [6] and "a reliable indicator of tobacco smoke exposure" [7]. These statements deserve further comment in the light of the recent finding of nicotine both in *Solanaceae* plants which are consumed as vegetables in our diet and in instant tea preparations [8]. Whilst nicotine, with the exception of carbon monoxide, is the most abundant single chemical in tobacco smoke, with an estimated yield of 1.0–2.3 mg per cigarette [9], with a mean nicotine intake per cigarette calculated as 0.75–1.25 mg per cigarette [10], it can no longer be considered as tobacco-specific. Significant nicotine concentrations (mg/kg dry weight) have been found in tomatoes (1.5–3.2), potato peel (9.5–16.1), eggplants (1.9–3.0), green peppers (1.3–3.9), green tea (1.8–2.4) and two brands of instant tea (12.2–28.0). It is possible to calculate from these data that a person who consumed 10 cups of tea in a day, ate food comprising 1 lb of tomatoes, aubergines or peppers and 1 lb of potatoes with their skins, might ingest nicotine equivalent to 1–2 cigarettes. The confounding effect, particularly in vegans and vegetarians, upon cotinine biomonitoring of environmental tobacco smoke exposure, might be considerable. It is hardly surprising that imported foodstuffs might contain high levels of nicotine, when nicotine is still widely used in the developing world as a cheap and effective insecticide. The data so far available though, suggest that

nicotine is elaborated by the *Solanaceae* such as tomatoes, since it is found not only in the fruit, but also the leaf, stem and root [8].

Whilst tobacco smoke contains high concentrations of nicotine, it also contains cotinine (9–57 μ g per cigarette) and nornicotine (27–88 μ g per cigarette) [4], both of which are mammalian metabolites of nicotine. Because nornicotine can be methylated in the lung to give nicotine [11], cotinine biomonitoring will reveal not only exposure to tobacco smoke and dietary nicotine, but also to cotinine itself and to nornicotine. Whether or not these latter two related alkaloids occur in vegetables or as degradation products of environmental nicotine insecticides is not known.

HUMAN METABOLISM OF NICOTINE AND COTININE AND ITS INTERSUBJECT VARIABILITY

It is generally assumed that cotinine arises from the metabolic oxidation of nicotine in human tissues by cytochrome *P*-450, although which discrete one of the myriad of *P*-450 isozymes effects this reaction is unclear. What is certain is that at any given time the concentration of cotinine in the plasma will depend upon not only the dose of nicotine ingested and inhaled, but is rate of conversion to cotinine, the rates of competing metabolic transformations to nornicotine and nicotine *N*-oxides, the rate of onward metabolism of cotinine to its own metabolites, together with both the rates of excretion of nicotine and cotinine in the urine and any sequestration of the two compounds within other body compartments which occurs. All these complex interactions are then candidates for intersubject variability arising from physiological, environmental, pathological and genetic differences which exist between all of us. What is so surprising, considering the perceived importance of nicotine, is that the balance of its metabolic transformations in man is not known: only a fraction of its metabolites have been identified.

For many years cotinine was considered to be the principal metabolite of nicotine and indeed many authors refer to it as such [10, 12–14]. However, recent studies point to *trans*-3'-hydroxycotinine as the major metabolite of nicotine [15]. Parviainen and Barlow [16] have criticized this interpretation of the chromatographic data and themselves refer to "metabolite 5" which behaves similarly to the

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trans-3'-hydroxycotinine of Neurath and Pein [15]. Further studies by these authors [17, 18] seem to establish the pre-eminence of *trans*-3'-hydroxycotinine amongst the known nicotine metabolites. Our own studies have shown that the *N*-oxides of nicotine are more abundant in the urine than cotinine after nicotine ingestion [19]. In studies where sufficient raw data were given to assess the extent of variability in cotinine levels under constant smoking conditions, the amount of intersubject variation observed is large. For example, the data cited by Neurath and Pein [15] for 9 subjects, who each smoked 19 cigarettes (each 1.35 mg nicotine) per day for 6 days, show that the plasma nicotine concentrations varied from 7–44 ng/ml, the cotinine from 41–344 ng/ml and the *trans*-3'-hydroxycotinine from 24–160 ng/ml, giving 6-, 8- and 7-fold variations respectively, with the greatest for cotinine. Similarly in the urinary studies of Cholerton *et al.* [19], cotinine excretion displayed the highest coefficient of variation of any metabolite. Cytochrome *P*-450-mediated metabolic oxidations are frequently subject to genetic polymorphism [20, 21] and as yet undefined genetic polymorphism may underlie this observed variation. What can be said is that plasma and urinary concentrations of cotinine do not reflect the dose of nicotine entering the body.

DISPOSITION OF COTININE IN SALIVA

In spite of the fact that a wide variety of drugs is subjected to therapeutic monitoring, for very few is this routinely done so in saliva instead of in plasma. It is only really for certain anti-convulsant drugs that the "non-invasive" salivary protocol has become a reality. It is noteworthy that Danhof and Breimer [22], in their review of therapeutic drug monitoring in saliva, conclude "In single dose studies, many discrepancies in the saliva/plasma ratio have been described, especially during periods of drug absorption. It is probably not possible to conduct reliable pharmacokinetic and biopharmaceutical single dose studies based upon saliva data alone". The subsequent decade has witnessed a gradual abandonment of the use of saliva in therapeutic drug monitoring. Nevertheless, salivary cotinine continues to be popular with the epidemiologists [23–25].

Two characteristics of a drug determine its penetration into saliva, its ionization constant expressed as a pK_a value and the fraction

unbound to plasma proteins. In the case of nicotine, both nitrogen atoms are ionizable with pK_a s of 8.02 and 3.12 [26], meaning that nicotine is both mildly and weakly basic. Cotinine however has lost its mildly basic pyrrolidine nitrogen to become a lactam, leaving only the weakly basic pyridine nitrogen with a pK_a of 4.37 [27].

Unlike nicotine, which is too basic, cotinine, with a pK_a of less than 5.5, is now able to freely enter saliva [28]. However, there is some contention that cotinine enters too freely, and is somehow concentrated by the gland, giving artificially high estimates of the plasma concentration [7], although this has been the subject of a strong debate [29, 30]. It is not unusual for drugs to be concentrated in saliva and this is one of the hallmarks of lithium which is actively transported into saliva to reach concentrations 2.2–3.3 times higher than plasma [22]. Active transport has also been proposed for both phenytoin and penicillin [22]. Accordingly either single spot concentrations or pharmacokinetic profiles of cotinine in saliva may over-represent the true disposition of the compound in plasma, thus clouding interpretation of such data.

ANALYTICAL DETERMINATION OF COTININE

A constellation of individual methodologies has been published. Many determine nicotine concentrations simultaneously. Many of the most sensitive, specific and reliable assays require the participation of a mass spectrometer [12, 31, 32] which is not available to most researchers. Gas chromatography using nitrogen-sensitive detection has also been employed [14, 33]. The ubiquitous HPLC has been applied to the problem [13, 16, 34, 35]. Probably the biggest advance has been the appearance of an enzyme-linked immunosorbent assay (ELISA) using a monoclonal antibody which recognizes cotinine [36]. Although only minimal cross-reactivity with nicotine and nicotine metabolites occurs, until the discovery by Neurath and Pein [15] that *trans*-3'-hydroxycotinine was the principal metabolite of nicotine hitherto, no assessment of cross-reactivity between the commonly-used anticotinine antibodies and *trans*-3'-hydroxycotinine was performed. Indeed this major metabolite cross-reacts by about 30% with the polyclonal rabbit anticotinine antiserum commonly used to determine cotinine levels by ELISA [37].

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USES AND ABUSES OF COTININE

In situations where the cotinine concentrations are massively elevated, such as in the urine, blood and saliva of active smokers, cotinine determinations should in principle serve a useful purpose in verifying self-reported smokers and non-smokers. The experiences of Jarvis *et al.* [24] are salutary in this regard. These investigators studied 215 outpatients attending cardiology and peripheral vascular clinics where a reasonably high proportion of cigarette smokers were anticipated. Accurate self-reporting of smoking status was encouraged by guaranteeing confidentiality and anonymity of responses. Eleven different biomarkers of smoking were determined on each subject including expired carbon monoxide and %COHb; plasma, saliva and urine thiocyanate, nicotine and cotinine. Plasma cotinine gave the best discrimination between self-reported smokers and non-smokers, but nevertheless 21 self-reported non-smokers had plasma cotinine levels similar to the smokers (cut-off 13.7 ng/ml cotinine). These 21 anomalous individuals were labeled "Deceivers" by these authors, even though 5 of them did not have raised thiocyanate levels and 3 had expired CO levels below the smoking cut-off of 5.6 ppm. Presumably the anonymity also protects the authors from litigation. What is most surprising is that no comment is made by the authors about the 9 self-reported smokers who had plasma cotinines below the cut-off of 13.7 ng/ml. In spite of its better performance than other biomarkers tested, plasma cotinine could still be seriously wrong on an individual case basis in this study. Using pejorative terminology such as "deceiver" to label a patient as a liar is in my view not only highly invasive of that patient's human rights but also unscientific. It may very well be that, by trading knowledge for convenience, an unusually high dietary intake of nicotine and cotinine has been overlooked, or a pharmacogenetic variant in nicotine/cotinine metabolism has stealthily been at work, or for that particular sample the assay did not perform adequately. This may be particularly important when issues of minor nicotine intake, such as environmental tobacco smoke, are being considered, where the signal-to-noise for cotinine values might be seriously confounded and compromised by diet and pharmacogenetic variation. Again the literature contains such possible examples: 330 non-smoking adolescent

schoolgirls in south London were studied by Jarvis *et al.* [38]. They were partly categorized as non-smokers by having salivary cotinine concentrations of less than 14.7 ng/ml [24]. The authors claim that the high correlation ($r = 0.75$, $p < 0.0001$) between non-smoking girls' salivary cotinine concentrations on two occasions 1 year apart was due to a constant home environment where either or both parents smoked indoors. Thus, the pattern of salivary cotinine due to the breathing of one or both parents' smoke was maintained with high reproducibility over 12 months. At least this is the interpretation put on the findings by the authors and they may be right, but they have ignored the possibility that an individual's salivary cotinine level is fixed by a combination of environmental, physiological and genetic factors and not merely by one exposure factor. From my perspective as a pharmacogeneticist a great deal of work is yet required before such weight can be attributed to salivary cotinine concentrations as is often witnessed. The full metabolic picture of nicotine is not known, the extent to which single genes can determine individual patterns is unknown, the complete dietary spectrum of nicotine and related pyridine alkaloids is unavailable at present, and the ability of salivary glands to concentrate cotinine is still under debate. I believe that the time is right to trade some expediency for proper investigation of the problem.

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Toxicology Letters, 35 (1987) 45-52
Elsevier

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TXL 01711

DETERMINATION OF NICOTINE AND COTININE IN HUMAN SERUM AND URINE: AN INTERLABORATORY STUDY*

(Smokers; non-smokers; radioimmunoassay; gas chromatography; interassay
variation)

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(Received 15 September 1986)

(Accepted 25 September 1986)

SUMMARY

An interlaboratory study aimed at determining nicotine and cotinine in human serum and urine was carried out. 11 laboratories from 6 countries, all experienced in performing nicotine and cotinine determinations in biological fluids by radioimmunoassay (RIA) and/or gas chromatography (GC) were involved. Each of them received 18 serum and 18 urine samples. The specimens were obtained from 8 smokers and 10 non-smokers; 2 samples from non-smokers were spiked with defined amounts of nicotine and cotinine. All the laboratories distinguished perfectly between the smokers and the non-smokers and according to cotinine levels in serum the laboratories ranked the samples with good agreement. There were systematic differences in the absolute values between the laboratories. The ratios of urinary cotinine concentrations between active and passive smokers differed widely from laboratory to laboratory. The reasons for this are not yet known and necessitate further investigation.

*Presented at the International Experimental Toxicology Symposium on Passive Smoking, October 23-25, 1986, Essen (F.R.G.).

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Abbreviations: c.v., coefficients of variation; ETS, environmental tobacco smoke; GC, gas chromatography; RIA, radioimmunoassay.

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Elimination of Cotinine from Body Fluids: Disposition in Smokers and Nonsmokers

NANCY J. HALEY, PhD, DANIEL W. SEPKOVIC, PhD, AND DIETRICH HOFFMANN, PhD

Abstract: We have evaluated differences in the elimination of cotinine, a major nicotine metabolite, in smokers who quit smoking and never-smokers who were exposed to environmental tobacco smoke (ETS) under controlled conditions. The mean biological half-life of cotinine in urine, collected from the nine smokers was 16.5 ± 1.2 h, in never-smokers exposed to ETS, 27.3 ± 1.9 h. Differences in the mode of uptake and absorption of nicotine and possible differences in nicotine metabolism may play roles in the clearance rate differences between smokers and nonsmokers. (*Am J Public Health* 1989; 79:1046-1048.)

Introduction

The elimination of nicotine, by smokers, users of other tobacco products, and by nonsmokers exposed to environmental tobacco smoke (ETS), is a variable metabolic process.¹⁻³ In a previous study, we observed that cotinine elimination in nonsmokers exposed to ETS tended to take longer than that seen in active smokers who quit smoking.⁴ The slower clearance in nonsmokers of a terminal nicotine metabolite suggested that other tobacco smoke constituents or their metabolites might also remain in the bodies of nonsmokers for longer time periods.

Differences in the induction of hepatic and possibly pulmonary enzymes can play a role in the diverse metabolic rates observed between individuals.⁵⁻¹¹ Additionally, variations in the site and rate of absorption of nicotine can affect first pass metabolism by the liver and influence nicotine conversion as well as nicotine clearance.⁸⁻¹⁰ Therefore we designed the current study to evaluate possible differences in the rate of cotinine elimination in smokers and ETS-exposed never-smokers.

Methods

Nine smokers between 23 and 38 years of age abstained from cigarette smoking for the duration of the study. Their prior smoking behavior ranged from self-reported use of six to 30 cigarettes per day. Smoking status was confirmed by urine cotinine concentrations. All urine voids were collected in four daily aliquots for five days beginning with cessation of smoking.

Ten nonsmokers who reported that they were never-smokers (26-45 years of age) were exposed to ETS for 80 minutes twice daily in two successive days in a specially constructed chamber. Five subjects were exposed at each time to the sidestream smoke produced by four Kentucky 1R1 Reference cigarettes which were continuously smoked

throughout the exposure period. The chamber conditions have been described previously.¹² Briefly summarized, the chamber is 16.3 m³ with six air changes per hour which corresponds to the average ventilation condition of offices in the United States. The pollution levels of selected smoke constituents were approximately 25 ppm CO, 0.9 ppm NO_x, and 160 µg/m³ formaldehyde. These are considered high pollution levels. Urine samples were collected before exposure and in four daily aliquots as described for the smokers following cessation.

Each sample was analyzed for cotinine by radioimmunoassay (RIA) using specific antisera produced in rabbits.^{13,14} Creatinine in urine was determined on a Kodak Ektachem 400 Clinical Chemistry Analyzer.¹⁵ Creatinine excretion was used to normalize urine cotinine values.¹⁴

The volumes of smoke exhaled during smoking or ETS exposure are variable as well as the efficiency of lung clearance. These factors and the amount of matter expectorated or swallowed and absorbed from the digestive tract will affect bioavailability. In light of these variables, calculations of the dose of smoke taken up by subjects would be inappropriate. We have therefore assessed exposure by terminal cotinine half-lives ($t_{1/2}$) estimated by linear regression of the log concentrations against time using an interactive computer program designed for the analysis of drug pharmacokinetics.¹⁶

Results

Data on the elimination of cotinine in the urine of smokers and ETS-exposed nonsmokers are presented in Table 1. The mean of the $t_{1/2}$ for cotinine in the urine of the nine smokers was 16.5 ± 1.2 h. The coefficients of variation between subjects was 21 percent. Nonsmokers who were exposed to ETS had a mean $t_{1/2}$ for cotinine elimination of 27.3 ± 1.9 h. The correlation coefficient for goodness of fit was $r = 0.89 \pm 0.02$ in smokers and $r = 0.90 \pm 0.01$ for nonsmokers exposed to ETS. For both smokers and nonsmokers, no correlation was observed between peak cotinine levels and elimination time. Nonsmokers had a substantially longer mean $t_{1/2}$ of cotinine elimination (27.3) than smokers who quit (16.5).

Discussion

The results of this study support the concept that cotinine elimination can be more rapid in smokers than in nonsmokers who are exposed to ETS, and the earlier work by Kyerematen, *et al*, who observed that the $t_{1/2}$ of nicotine in the urine of nonsmokers was more than twice as long as that of smokers.¹⁷ Taken together, these observations could reflect a heightened carcinogenic potential of ETS in nonsmokers.

The slower clearance in nonsmokers of a terminal nicotine metabolite suggests that other tobacco smoke constituents or their metabolites may also remain in the bodies of nonsmokers for longer periods of time. Conceivably, this could increase the potential for endogenous formation of carcinogenic tobacco specific nitrosamines.⁸ In considering

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TABLE 1—Cotinine Elimination in Urine of Smokers and ETS-Exposed Nonsmokers

Subject	t ^{1/2} (h)	r	Y intercept (ng/mg creatinine)	Peak Cotinine Levels (ng/mg creatinine)
Smokers				
A	15.3	0.90	2508	7317
B	13.6	0.95	167	268
C	12.1	0.92	1368	1904
D	18.7	0.89	3008	2668
E	17.4	0.96	348	1822
K	17.5	0.96	908	839
L	17.6	0.94	48	72
M	23.3	0.81	215	207
N	13.5	0.94	1417	2368
Mean \pm (SEM) 16.5 \pm 1.2 (95% CI = 13.6, 19.6)				
Nonsmokers				
F	28.7	0.89	29	25
G	23.3	0.96	62	96
H	19.9	0.94	14	34
I	33.4	0.87	20	64
J	21.4	0.86	24	66
O	18.8	0.96	43	59
P	32.3	0.94	41	91
Q	31.9	0.87	33	31
R	34.0	0.90	112	117
S	28.8	0.91	19	24
Mean \pm (SEM) 27.3 \pm 1.9 (95% CI = 25.3, 29.4) ^{a,b}				

^aSignificantly different from smoker group ($p < 0.0007$).

^bThe confidence interval for the difference between means (10.8 h) was determined by first obtaining a pooled estimate of the variance. In doing both confidence limits were positive (95% CI = 1.4, 20.2), we concluded that the means between the groups were different.

the toxic burden imposed by exposure to cigarette smoke, it is important to note that sidestream smoke which is a major fraction of ETS contains higher levels of certain toxic cocarcinogenic and cancerogenic compounds than does mainstream smoke.¹⁸ Before dilution with room air, some of these compounds are 50- to 100-fold higher in the sidestream smoke compared to their concentration in mainstream smoke.^{19,20}

It is well documented that many compounds contained in tobacco smoke induce hepatic and pulmonary mixed function oxidases, yet the magnitude and duration of induction remains unclear.^{14,21} It is conceivable that inhaled mainstream smoke and ETS might have varying effects on liver or lung oxidases due to the varying concentrations of smoke constituents. The importance of lung metabolism of inhaled xenobiotics is recognized.²²

Differences between smokers and nonsmokers in the metabolism of drugs are substantial. Early studies by Hart, *et al.*, have demonstrated enhanced drug metabolism in smokers by using anti-pyrene as an index of hepatic metabolism.²³ Powell, *et al.*, reported a theophylline half-life intermediate between smokers and nonsmokers in ex-smokers who abstained for three years.²⁴ Thus, differential rates of metabolism of nicotine in smokers and nonsmokers might be predicted from earlier work.

In our study, the $t_{1/2}$ in never-smokers exposed to ETS under strictly controlled conditions was significantly longer than that seen for smokers. In most cases, the peak cotinine levels for smokers were much higher than in the ETS-exposed subjects, but no relation existed between peak cotinine levels and the $t_{1/2}$ for cotinine excretion. It is

probable, therefore, that elimination rate is not as greatly affected by dose as by method of exposure or subject metabolism.

A recent study by Jarvis, *et al.*, concluded that differences in cotinine elimination do not exist between smokers and never-smokers.²⁵ In that study, nicotine pellets were ingested by five subjects of mixed smoking habits (cigars, cigarettes, and ex-smokers). Since such a protocol fails to allow for possible metabolic differences between smokers and never-smokers, ignores the lung as a metabolic site, and precludes the possibility of synergistic effects of any of the more than 3,800 constituents in tobacco smoke, we feel that the data of Jarvis cannot be compared to the current controlled study of cotinine elimination following active smoking or ETS exposure in smokers and never-smokers.

ACKNOWLEDGMENT

We would like to thank Carys M. Axelrod and Stephen Coleman for performing the cotinine analyses. We gratefully acknowledge Ilse Hoffmann's editorial assistance.

This work was supported by National Cancer Institute grants P01-CA 29580 and P01-CA 32617.

These data were presented, in part, at the International Symposium of Nicotine, Queensland, Australia, September, 1987.

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Use of Smokeless Tobacco, Cigarette Smoking, and Hypercholesterolemia

LARRY A. TUCKER, PhD

Abstract: The primary purpose of this study was to determine the extent to which regular use of smokeless tobacco is associated with hypercholesterolemia (≥ 6.2 mmol/L) among 2,840 adult males. The confounding effects of age, education, physical fitness, body fatness, and other tobacco use were also examined. After adjustment, smokeless tobacco users were 2.5 times, heavy smokers were 2 times and mild/moderate smokers were 1.5 times more likely to have hypercholesterolemia than non-users of tobacco. Cigarette smokers did not differ significantly from users of smokeless tobacco regarding hypercholesterolemia. Users of smokeless tobacco were younger and less educated compared to non-users of tobacco, while smokers were less educated and less physically fit. (*Am J Public Health* 1989; 79:1046-1050.)

Introduction

National estimates indicate that at least 12 million Americans used some form of smokeless tobacco during 1985 and recent data show that 16 percent of males 12 to 25 years of age have used smokeless tobacco within the past year. An estimated 6 million persons use smokeless tobacco at least weekly and rates seem to be increasing, especially among adolescent and young adult males.¹

The increased appeal and use of smokeless tobacco has generated considerable public health concern because research indicates that snuff and chewing tobacco can be significant health hazards. Most notably, dipping and chewing have been linked to oral cancer,¹⁻³ and numerous clinical studies have shown strong associations between smokeless tobacco use and noncancerous and precancerous oral conditions.⁴⁻⁵

Many of the health problems associated with tobacco use are a consequence of nicotine. Since the blood nicotine levels which result from smokeless tobacco use are similar to those from cigarette smoking,⁶⁻¹¹ the nicotine-related health effects of smoking would also be expected to result from using smokeless tobacco. Compared to nonsmokers, smokers tend to have elevated levels of low density and very low density lipoproteins and reduced levels of high density lipoproteins,¹²⁻¹⁷ a lipid profile strongly associated with increased risk of atherosclerosis and coronary heart

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disease.¹⁸⁻²² To date, only one unpublished study has been conducted to determine the effects of smokeless tobacco use on lipid metabolism.²³

The present study was conducted to determine the extent to which use of smokeless tobacco contributes to hypercholesterolemia controlling for lifestyle and demographic factors, and to compare the effects of smokeless tobacco and cigarettes on hypercholesterolemia.

Methods

Subjects

A sample of 2,840 adult males with a mean age of 40.7 ($SD = 10.8$) was studied. Subjects were employees of over 25 different companies that participated in the Health Examination Program offered by Health Advancement Services (HAS), Inc. Approximately 70 percent of the men were married, 76 percent were White, and 73 percent had some college education. The median and modal annual gross family income was \$25,000-\$30,000.

All data were collected by registered nurses employed by HAS, Inc. Each subject was examined individually and privately for approximately 60 minutes after participating in an orientation and completing an informed consent form.

Instrumentation and Procedures

A written questionnaire was administered to assess demographic and life-style information, including use of smokeless tobacco and cigarettes. A Harpenden skinfold caliper was employed to assess subcutaneous fat at three body sites and the sum of the skinfold measurements along with age and gender were used to calculate the total body fat percentage of each subject.²⁴ Physical fitness was assessed using a step test, the Raach 3-minute Pulse Recovery Test.²⁵ Approximately 10cc of blood was drawn from each subject and analyzed using the enzymatic method to determine serum cholesterol levels.²⁶

Data Analysis

Subjects were classified as regular users of smokeless tobacco, mild/moderate smokers (1-20 cigarettes/day), heavy smokers (>20 cigarettes/day) or non-users of tobacco, according to their questionnaire responses. High blood cholesterol or hypercholesterolemia was defined as a total serum cholesterol level of 6.2 mmol/L or greater, consistent with the criteria of the National Cholesterol Education Program New Cholesterol Adult Treatment Guidelines.²⁷ The control variables were categorized as depicted in Table 1.

The associations between smokeless tobacco use, cigarette smoking, and hypercholesterolemia were measured by the odds ratio.²⁸ To control for potential confounders, Mann-

Address reprint requests to Larry A. Tucker, PhD, Associate Professor, Division of Health Promotion, 275 STH, Brigham Young University, Provo, UT 84602. This paper, submitted to the Journal July 27, 1988, was revised and accepted for publication February 10, 1989.

INTRODUCTION

Nicotine and cotinine in body fluids are often used in order to quantify tobacco smoke uptake. For their determination, GC and RIA methods are normally applied. A controversial discussion in the literature gives rise to some doubt whether data published by different laboratories are comparable with one another [1, 2]. The analytical difficulties appear to be most evident in the case of ETS exposure, as very low exposure levels coming close to the detection limit have to be measured. In order to gain further insight into this matter, we initiated an international interlaboratory study. 11 laboratories were involved being experienced in performing nicotine and cotinine determinations in biological fluids by RIA and/or GC.

TABLE I

NICOTINE IN SERUM (ng/ml) AS MEASURED BY 5 DIFFERENT LABORATORIES USING GASCHROMATOGRAPHY (GC)

X, S and C.V. are calculated without the outlying measurements.

Subjects	Smoke uptake	Laboratories - Methods					\bar{x}	s	C.V.
		A-GC	D-GC	F-GC	H-GC	K-GC			
Non-smoker	ETS exp. ^b								
1	0	<1	6.8 ^a	<0.5	0.4	1.0			
2	0	<1	4.3 ^a	<0.5	0.2	1.2			
3	1	<1	7.4 ^a	0.8	0.3	1.5			
4	2	<1	5.7 ^a	0.5	0.3	1.3			
5	2	<1	5.3	0.9	0.6	1.8			
6	3	<1	10.1 ^a	0.6	0.5	2.1			
7	3	<1	nd	<0.5	0.2	1.8			
8	3	1	9.6 ^a	0.8	0.3	2.1			
Smoker	Cig./day								
9	15	19	15.0	17.5	18.6	20.4	18.1	2.0	0.11
10	15	7	10.4	9.0	8.8	13.2	9.7	2.3	0.24
11	20	3	15.0 ^a	11.9	2.3	2.2	2.3	0.5	0.20
12	20	8	14.2	4.8	6.3	8.0	8.3	3.6	0.43
13	30	11	23.7	14.3	15.8	16.7	16.3	4.7	0.29
14	40	22	9.9	12.9	15.0	15.8	15.1	4.5	0.30
15	40	27	29.8	25.0	27.7	37.1	29.3	4.7	0.16
16	60	32	24.1	32.4	40.0	40.7	33.8	6.8	0.20
17 ^c		39	26.8	35.4	44.9	44.1	38.0	7.4	0.19
18 ^c		15	17.3	17.5	21.8	21.9	18.7	3.0	0.16

nd, not detectable.

^aOutlier.

^bETS exposure: 0, none; 1, low; 2, medium; 3, high.

^cSerum spiked with 41 and 20 ng/ml nicotine, respectively.

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MATERIALS AND METHODS

Urine obtained from 8 non-smokers and 8 smokers was collected for 24 h, and blood was drawn between 4 and 6 pm at the Forschungslaboratorium Prof. Schievelbein. 6 of the non-smokers reported exposure to ETS. The urine and serum specimens were aliquoted and stored at -20°C . In addition, 2 serum and 2 urine samples derived from non-exposed non-smokers were spiked with nicotine and cotinine. In total, there were 18 urine and 18 serum samples. The frozen specimens (4 ml serum and 10 ml urine) were dispatched on dry ice. The participating laboratories were sent the samples under a code number. Each laboratory could choose its own analytical method. They were asked to return one single value for nicotine and cotinine from each sample.

TABLE II

NICOTINE IN URINE (ng/ml) AS MEASURED BY 5 DIFFERENT LABORATORIES USING GASCHROMATOGRAPHY (GC)

X, S and C.V. are calculated without the outlying measurements.

Subjects	Smoke uptake	Laboratories - Methods					x	s	c.v.
		A-GC	D-GC	F-GC	I-GC	K-GC			
Non-smoker	ETS exp. ^b								
1	0	4	nd	8.3	<5	7.7			
2	0	7	5	4.0	26	16.0			
3	1	3	nd	4.5	<5	2.3			
4	2	<3	nd	4.9	450 ^c	6.2			
5	2	<3	nd	2.4	<5	8.4			
6	3	<3	nd	4.9	38	15.9			
7	3	9	nd	5.6	28	8.2			
8	3	32	nd	19.9	540 ^c	34.0			
Smoker	Cig./day								
9	15	518 ^a	149	124	150	190	153	27	0.18
10	15	1191	907	1190	345	1189	964	367	0.38
11	20	255	186	154	118	204	183	51	0.28
12	20	861 ^a	615	609	650	650	631	22	0.04
13	30	519	459	450	338	500	453	70	0.16
14	40	1333	1155	1092	1320	627	1105	286	0.26
15	40	3218	2967	3331	3198	3125	3168	134	0.04
16	60	3279	3417	3486	2813	3391	3277	270	0.08
17 ^c		2687	2483	2599	1365 ^a	2601	2592	83	0.03
18 ^c		1404	1112	1356	1025	1269	1233	161	0.13

^aOutlier. nd, not detectable.

^bETS exposure: 0, none; 1, low; 2, medium; 3, high.

^cUrine spiked with 2500 and 1200 ng/ml nicotine, respectively.

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RESULTS AND DISCUSSION

Only 5 laboratories were able to measure nicotine in serum and in urine, all of them using GC. Cotinine in serum and urine were each determined by 10 laboratories. The results are summarized in Tables I to IV. The recovery rates calculated from the spiked serum and urine samples ranged from 55 to 117% for

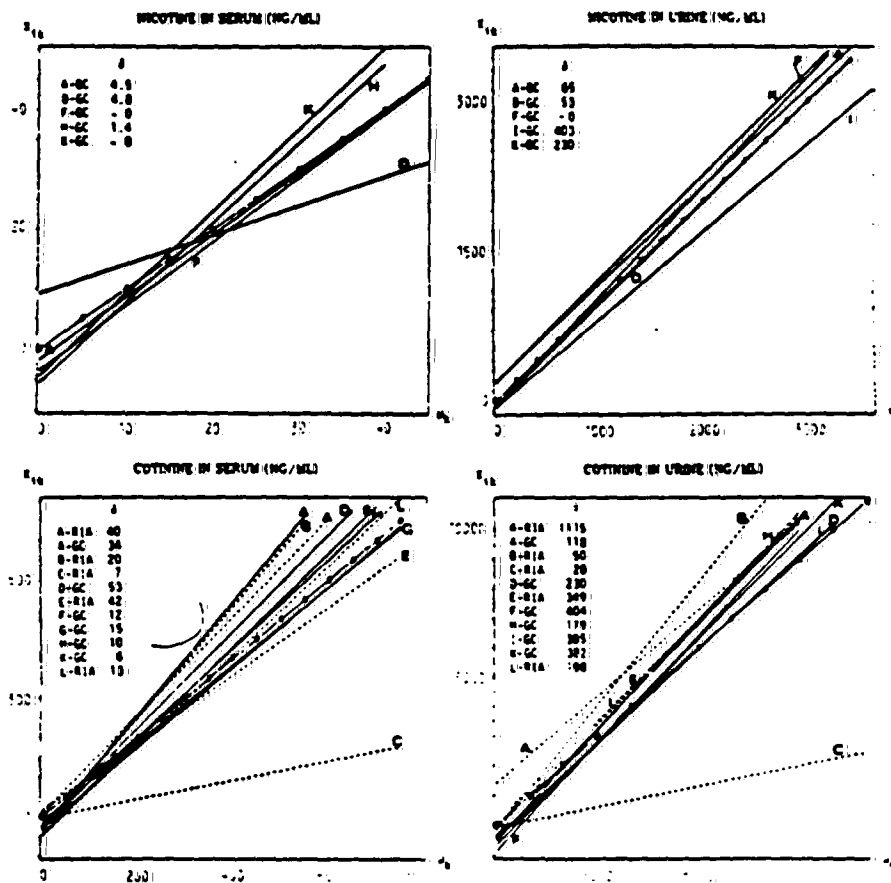


Fig. 1. Linear model (according to Jaech [3]) based on the samples from smokers and on the spiked samples ($n = 10$).

Model, $X_{ki} = \alpha_i + \beta_i \cdot \mu_k + \epsilon_{ki}; i = 1, \dots, 11; k = 9, \dots, 18.$

X_{ki} , measurement value for sample k , derived by laboratory i .

μ_k , true but unknown value for sample k .

(α_i, β_i) , bias for laboratory i .

ϵ_{ki} , error term with expectation 0 and variance σ_i^2 .

●—●, diagonal line in plots.

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TABLE III
COTININE IN SERUM (ng/ml) AS MEASURED BY 10 DIFFERENT LABORATORIES USING RADIOIMMUNOASSAY (RIA) AND/OR GAS
(CHROMATOGRAPHY) (GC)
x, y and c.v. are calculated without the outlying measurements.

Subjects	Smoker	Non-smoker	ETS exp. ^a	Laboratories - Methods										x	s	C.V.	
				A-RIA	A-GC	B-RIA	C-RIA	D-GC	E-RIA	F-GC	G-GC	H-GC	K-GC				L-RIA
1	0	<1	<1	0.6	nd	8.6	nd	2	62 ^b	<0.1	0.5	<1	<1	<1	<1	<1	<1
2	0	<1	<1	0.6	nd	3.0 ^b	nd	<1	nd	<0.1	0.8	<1	<1	<1	<1	<1	<1
3	1	<1	<1	2.0	nd	6.4 ^b	nd	<1	nd	1.4	1.4	<1	<1	<1	<1	<1	<1
4	2	2	2	0.8	nd	2.3	nd	<1	25 ^b	0.2	0.6	<1	<1	<1	<1	<1	<1
5	2	2	2	0.9	nd	3.0	nd	2	nd	<0.1	0.5	<1	<1	<1	<1	<1	<1
6	3	6	6	6.4	0.2	7.7	nd	4	15	2.5	5.5	<1	<1	<1	<1	<1	<1
7	3	2	2	1.9	0.4	nd	nd	4	nd	0.4	2.4	<1	<1	<1	<1	<1	<1
8	3	4	4	4.9	nd	5.1	nd	3	nd	2.1	3.5	<1	<1	<1	<1	<1	<1
9	15	228	260	211	39.7	196	119	179	165	198	171	175	176	58	0.33	176	0.33
10	15	80	107	90	12.0 ^b	84	65	73	67	75	67	57	76	14	0.19	76	0.19
11	20	60	75	63	11.7	40	40	31	47	45	42	14	42	19	0.45	42	0.45
12	20	338	229	234	41.0 ^b	197	199	157	155	179	172	168	202	54	0.27	202	0.27
13	30	330	289	325	53.3 ^b	370	289	250	210	242	228	251	278	51	0.18	278	0.18
14	40	357	354	334	54.2 ^b	260	263	261	222	251	255	231	278	50	0.18	278	0.18
15	40	620	741	670	105.8 ^b	680	449	587	510	555	498	544	585	92	0.16	585	0.16
16	60	631	697	706	120.6 ^b	481	382	527	480	529	467	525	542	104	0.19	542	0.19
17	457	463	478	478	95.0 ^b	460	388	398	330	399	347	351	407	54	0.13	407	0.13
18	223	224	249	249	42.5 ^b	220	208	185	165	196	198	183	205	24	0.12	205	0.12
nd, not detectable.																	

nd, not detectable.

^aQuitter.

^bETS exposure: 0, none; 1, low; 2, medium; 3, high.

^cSerum spiked with 420 and 210 ng/ml cotinine, respectively.

TABLE IV

COTININE IN URINE (ng/ml) AS MEASURED BY 10 DIFFERENT LABORATORIES USING RADIOIMMUNOASSAY (RIA) AND/OR GAS CHROMATOGRAPHY (GC)

x, s and c.v. are calculated without the outlying measurements.

Subjects	Smoke uptake	Laboratories - Methods											x	s	c.v.
		A-RIA	A-GC	B-RIA	C-RIA	D-GC	E-RIA	F-GC	H-GC	I-GC	K-GC	L-RIA			
Non-smoker	ETS exp. ^a														
1	0	7	13	3.9	18.2	nd	nd	18		18	3.4	<1			
2	0	25	2	13.0	4.0	43	nd	10		26	15.7	4.0			
3	1	17 ^a	5	4.5	nd	nd	nd	4		7	4.9	1.0			
4	2	17 ^a	2	4.4	3.1	nd	nd	7		8	2.9	<1			
5	2	11	2	2.6	14.5	nd	nd	2		30 ^a	3.6	<1			
6	3	76	38	45.0	36.1	nd	44	21		19	30.6	12.6			
7	3	31	19	16.0	nd	nd	nd	9		12	8.5	3.8			
8	3	150 ^a	38	75.0	45.9	nd	61	15		61	21.4	29.1			
Smoker	Cig./day														
9	15	2153	1298	1635	268	1150	1541	878	1370	890	1050	1236	1223	484	0.40
10	15	1002	1014	740	173	512	1080	460	720	903	605	538	704	279	0.40
11	20	626	324	425	66	205	301	129	190	178	201	260	264	154	0.59
12	20	5196 ^a	1749	2630	302	1145	2979	1002	1590	1420	1091	2580	1648	847	0.51
13	30	3726 ^a	1124	2015	232	700	1823	732	940	690	899	1474	1063	554	0.52
14	40	3726	1736	2550	347	1393	2355	703	1570	1750	745	2416	1754	979	0.56
15	40	4040	2536	3490	486	2253	2960	2091	2430	1565	2270	2604	2429	934	0.38
16	60	5245	3512	4120	671 ^a	3193		2833	3280	3278	2920	3718	3566	742	0.21
17 ^a		8478	8623	10240	1645 ^a	7464	8774	8236	8710	7400	7997	8500	8442	798	0.09
18 ^a		4088	4120	4970	795 ^a	3573	3834	4159	4270	4108	4018	4242	4138	357	0.09

nd, not detectable.

^aOutlier.

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nicotine, and from 79-119% for cotinine. There were no differences in recovery rates between the urine and serum samples. The data from laboratory C were excluded from this calculation, as they show a recovery rate for cotinine of about 20% only.

In the samples obtained from smokers the interlaboratory c.v. ranged from 4 to 59%. They were not influenced by increasing cigarette consumption. With regard to nicotine, the coefficients of variation were similar when the measurements were made in serum or urine, whereas for cotinine the mean coefficient of variation in urine was twice as high as found in serum. This is due to the fact that cotinine determinations in urine by RIA are less precise than in serum. For the spiked samples the coefficients of variation ranged from 3 to 19% being much lower than in the samples from smokers. As yet we have no explanation for this. The coefficients of variation for the samples obtained from non-smokers should not be calculated, since most of the values are below the detection limit and no numerical figures are available. The ratios of urinary cotinine concentrations between active and passive smokers differed widely ranging from 21 in laboratory C to 294 in laboratory L.

In order to describe the results of the interlaboratory study in a more condensed form, a linear model [3] based on the samples obtained from smokers and on the spiked samples was used (Fig. 1). A comparison of the model line obtained from each laboratory with the ideal line (diagonal) indicates the degree of deviation of the laboratory's results. A low σ -value means high precision. Cotinine values in serum are comparable whether determined by RIA or GC, whereas in urine the RIA values are higher than the GC values. The data obtained in laboratory C have been disregarded.

The results of our interlaboratory study indicate that data on nicotine and cotinine concentrations in serum and urine from smokers published so far are certainly comparable on a relative basis (coefficient of correlation: 0.6-0.9). In general, the laboratories ranked the samples according to cotinine levels in serum with good agreement. The absolute values, however, show large interlaboratory variations. These are particularly high in the samples obtained from subjects exposed to ETS.

ACKNOWLEDGEMENTS

The study was carried out in the following laboratories: Dr. N. Benowitz, Clinical Pharmacology Unit, General Hospital Center, San Francisco (U.S.A.); Dr. A. Biber, Forschungslaboratorium Prof. Schievelbein, Munich (F.R.G.); Dr. M. Curvall, Swedish Tobacco Co, Stockholm (Sweden); Dr. C. Feyerabend, Dr. M.A.H. Russell, Institute of Psychiatry, University of London, London (U.K.); Dr. J.E. Haddow, Dr. G.E. Knight, Foundation for Blood Research, Scarborough, ME (U.S.A.); Dr. N.J. Haley, American Health Foundation, New York (U.S.A.); Dr. S. Matsukura, Miyazaki Medical College, Kiyotake (Japan); Dr. H. Muranaka, Kyoto Senbai Hospital, Kyoto (Japan); Dr. H. Nau, Institut für Toxikologie und

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Embryonalpharmakologie, Freie Universität Berlin, Berlin (Germany); Dr. K. Engström; Dr. M. Sorsa, Institute of Occupational Health, Helsinki (Finland); Dr. N. Wald, Medical College of St. Bartholomew's Hospital, London (U.K.).

The authors are indebted to all the scientists participating in this study. Without their spontaneous cooperation it would not have been possible to carry out this investigation.

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2023381100

Measurement of Current Exposure to Environmental Tobacco Smoke

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ABSTRACT. Reports of recent exposure to environmental tobacco smoke (ETS) and urinary cotinine levels were obtained on 663 never- and ex-smokers who attended a cancer screening clinic in Buffalo, New York, in 1986. Study objectives included determining the prevalence of exposure to ETS using urinary cotinine and identifying questionnaire exposure measures predictive of cotinine. Findings demonstrate that exposure to environmental tobacco smoke is extremely prevalent, even among those not living with a smoker. A total of 76% of subjects reported exposure to ETS in the 4 d preceding the interview. The most frequently mentioned sources of exposure were at work (28%) and at home (27%). Cotinine was found in the urine of 91% of subjects. Cotinine values increased significantly with the number of exposures reported. Among the different questionnaire measures of exposure that were evaluated, the single best predictor of cotinine was the number of friends and family members seen regularly by the subject who smoke.

EXPOSURE to environmental tobacco smoke (ETS) has been implicated as a cause of many adverse health consequences in nonsmokers.¹⁻³ Although the health risks associated with ETS are probably small in com-

parison to active smoking, given the high prevalence of smoking in the United States,⁴ exposure to ETS is likely to be common, and the number of people adversely affected could be substantial.

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Health risks associated with ETS vary with exposure. Biological markers of tobacco smoke exposure have recently been used in an attempt to measure smoke absorption in nonsmokers.^{1,2,5-10} Of the various biochemical markers of tobacco smoke exposure, there is general agreement that cotinine is the best marker. Cotinine is a metabolite of nicotine and is therefore specific to tobacco smoke. Cotinine is considered a better marker of exposure than nicotine because its longer half-life means it measures exposure over several days rather than hours.⁶⁻¹⁰ The degree of exposure, as measured by cotinine, is likely to depend upon several environmental factors, including number of exposures, duration of exposure, intensity of exposure (i.e., number of smokers), and room ventilation characteristics.^{1,10}

This study examines the relative importance of these environmental factors in predicting current ETS exposure, as measured by urinary cotinine, in a group of 663 non-smokers. In addition, information is presented on the prevalence and sources of exposure to tobacco smoke. The findings from this study should be useful to those interested in developing valid questionnaire measures of recent exposure to ETS and to health officials charged with assessing the need for regulations that restrict smoking in public places.

Materials and methods

The study population included adult men and women who attended the Roswell Park Memorial Institute Cancer Screening Clinic for a free cancer check-up during 1986. Screening exams were performed by a nurse practitioner or physician. Following the examination, clinic attendees were asked by the examiner if they wished to participate in a study on ETS. Subjects were informed that participation in the study would require a 30- to 40-min interview, provision of a urine sample, and a lung function test. Among those approached, about 70% volunteered to participate. The main reason given for refusal to participate was lack of time. Those willing to participate were directed into a private office where an interviewer provided a further description of the study and obtained a signed consent.

Interviewing began in February 1986 and ended in December 1986. Smokers and non-smokers were enrolled in the study up until July 1986, after which time only non-smokers were recruited. A total of 860 individuals were enrolled in the study. These included 380 never-smokers, 350 ex-smokers, and 130 current smokers. Subjects were classified as ex-smokers if they smoked at least one cigarette/pipe/cigar a day for ≥ 1 yr and had not used tobacco for at least 1 mo prior to the interview. Current smokers were classified as those subjects who currently smoked any quantity of tobacco. The data presented in this paper are restricted to never- and ex-smoker participants in the study ($N = 730$).

The age distribution of subjects was widespread, ranging from 18 to 84 yr (mean age = 54.7 yr). Ex-smokers were slightly older (56.8 yr) than never smokers (53.2 yr). A higher proportion of never smokers compared to ex-smokers were below age 40 yr (21% vs. 12%). Overall, 44% of subjects were male,

and 90% were white. A significantly higher proportion of ex-smokers were male compared to never-smokers (55% vs. 34%). The majority of subjects were married (69%). Slightly more than one-third of never smokers were college graduates, in contrast to 25% among ex-smokers. Roughly half the study subjects were currently employed. In comparison to the adult population in Erie County, New York, the study sample over represented females and whites and under represented persons below 40 yr of age.

Data collection. Study subjects were interviewed in a private office by a trained interviewer. The interviewer questioned subjects about their current and past tobacco use habits, exposure to tobacco smoke at home and at work, and recent indoor exposure to tobacco smoke over a 4-d period preceding the interview. To aid recall, each of the 4 d was subdivided into three segments (i.e., morning, afternoon, evening), and subjects were asked the same questions for each portion of the day. For each portion of a day, subjects were asked to indicate whether they had been exposed indoors, not in a car, to smoke from an individual who was smoking. Those who answered "yes" were asked to report on the location and duration (measured in quarter hours) of exposure, the number of smokers present (within 10 ft), the size of each exposure location, and the air ventilation characteristics of each location (i.e., open windows, air conditioning). Subjects were asked to rate the size of each exposure location on a 3-point scale as follows: 1 = large, defined as auditorium size; 2 = medium, defined as kitchen or living room size; and 3 = small, defined as small, single-person office size.

For each portion of a day, subjects were also asked to indicate whether they had been exposed to one or more people smoking in a car. Those who answered "yes" were asked how many people were smoking, the duration of exposure (measured in quarter hours), and whether windows were open or air conditioning was being used. The location size score for exposures in a car were automatically coded a 3, which corresponded to a small indoor exposure location.

The recall interview was structured so that for each portion of a day the subject could report on a single indoor exposure and one exposure occurring in a car. Thus, the maximum number of exposure events that could be recorded in a given day was 6 (3 segments in a day \times 2 exposures per segment), and 24 for the entire 4-d recall period. If multiple exposure locations were reported in the same portion of the day, the more extensive exposure was recorded. Very few subjects reported multiple exposure locations during the same portion of a given day. Thus, the exposure reports recorded represent a fairly complete picture of a subject's perception of exposure to tobacco smoke over the 4 d preceding the interview.

In addition to information on tobacco smoke exposure, subjects were questioned about their current and past health status, work history, and personal characteristics. All subjects were given a lung function test and asked to provide a 6-ml urine sample for determination of cotinine.

Urine specimens were frozen at -80°C until the cotinine assay was performed. Assays were done within 6 mo of collection and without knowledge of the subject's smoking or exposure status. Cotinine was quantified using high pressure liquid chromatography (HPLC).¹¹ To check the accuracy of the assay, control samples with established mean cotinine values were performed with each HPLC run. If control values could not be repeated (i.e., coefficient of variation $> 10\%$), the assay was redone.

Exposure measures. Several indicators of exposure to ETS were constructed from interview responses. From the information collected in the 4-d recall, five measures of ETS exposure were computed. These included (1) the total number of exposures, computed by summing the number of indoor and car exposures reported by subjects; (2) total duration of exposures, computed by summing across all reported exposures, the number of minutes exposed to tobacco smoke; (3) the intensity of exposures, measured by summing across all reported exposures, the number of smokers to whom the subject was exposed within 10 ft; (4) the size of exposure locations, computed by summing the size scores across all exposures; and (5) the ventilation characteristics of exposure locations measured by having subjects indicate for each location whether it was ventilated (i.e., open windows or air conditioning) or not (ventilated = 1, not ventilated = 0), and then summing the ventilation scores across all exposures.

In addition to measures derived from the 4-d recall, several general indicators of current ETS were assessed. These included (1) the number of cigarette/cigar/pipe smokers living in the subject's home (coded as none, one, two, or more); (2) among married subjects, the smoking status of their spouse; (3) among currently employed subjects, exposure to tobacco smoke at work; and (4) a rating by subjects of the number of people they see regularly (i.e., friends, relatives, co-workers) who smoke (response categories were none/few, some, most/all).

Data analysis. Analyses were restricted to lifelong non-smokers and ex-smokers. Two subjects who used chewing tobacco were excluded from analyses. Other exclusions included 45 subjects from whom urine samples were not obtained or were lost; 14 subjects for whom the cotinine assay was judged to be unreliable, i.e., coefficient of variation greater than 10%; and 6 subjects whose cotinine levels exceeded 90 ng/ml and were, therefore, classified as active smokers. The cut-point of 90 ng/ml to distinguish between active and passive smoking was based on a comparison of the distributions for reported non-smokers and current smokers. Subjects excluded from the analysis did not differ significantly from those retained in the analysis with regard to demographic characteristics or self-reports of exposure to ETS.

The bivariate relationship between urinary cotinine and measures of ETS smoke exposure were evaluated by either one-way analysis of variance or Pearson Product Moment correlation coefficients, as appropriate. Multiple regression analysis was employed to evaluate the relationship between urinary cotinine and meas-

ures of exposure to passive smoke, controlling for the following potential confounding variables: age; sex; time of day when the specimen was collected (coded as: morning, afternoon, evening); and time of year when the specimen was collected (coded as: indoor months = November through April and outdoor months = May through October).

One-way analysis of variance was used to evaluate the relationship between the characteristics of subjects and exposure to ETS as measured in the 4-d recall. A stepwise multiple regression analysis was performed to assess the multivariate importance of variables found in the bivariate analysis to be associated with reported exposures.

Results

Seventy-six per cent (501/663) of the subjects reported exposure to tobacco smoke in the 4 d preceding the interview. The average number of exposures reported over the 4-d period was 3.3 (range: 0 to 21 exposures). Among the 501 exposed subjects, the average daily exposure was 2 h (range < 1 h to 13.25 h/d). Reported exposure locations in order of frequency were work (28%), home (27%), restaurants (16%), private social gatherings (11%), in a car or airplane (10%), and in public buildings (8%).

Twenty-two per cent of subjects ($n = 145$) lived with a smoker. Of the 466 married subjects, 94 reported that their spouse smoked. Among currently employed subjects ($n = 343$), 77% reported being exposed to tobacco smoke at work. Twenty per cent of subjects stated that smoking is prohibited in their home; 40% prohibited smoking in their car. Fifty-seven per cent of subjects reported that none or few of their family and friends smoke, 29% said that some smoke, and 14% said that most or all smoke.

Six hundred and five of the 663 (91%) had detectable cotinine levels. The mean cotinine level was 8.84 ng/ml (median = 6.19 ng/ml). Cotinine levels ranged from 0 ng/ml to 85 ng/ml; 92% of cotinine values were less than 20 ng/ml.

Figure 1 shows the mean urinary cotinine levels by the number of exposures reported by subjects during the 4-d recall period. Whereas concentrations of cotinine varied widely within exposure groups, the level increased with the number of exposures reported (Pearson Product Moment Correlation = 0.23, $p < .01$).

Table 1 shows the relationship between urinary cotinine and various measures of exposure to ETS computed from the 4-d recall portion of the interview. All exposure measures were significantly related to cotinine, although the degree of association was modest. Exposure measures were highly intercorrelated because each was based on the number of exposure occurrences (range: $r = 0.70$ to $r = 0.96$). To evaluate the relationship of exposure duration, intensity, room size, and room ventilation with cotinine, independent of number of exposures, partial correlations were computed controlling for number of exposures. The partial correlation coefficients are shown in the second column in Table 1. When the number of exposure occurrences was controlled, only the ventilation character-

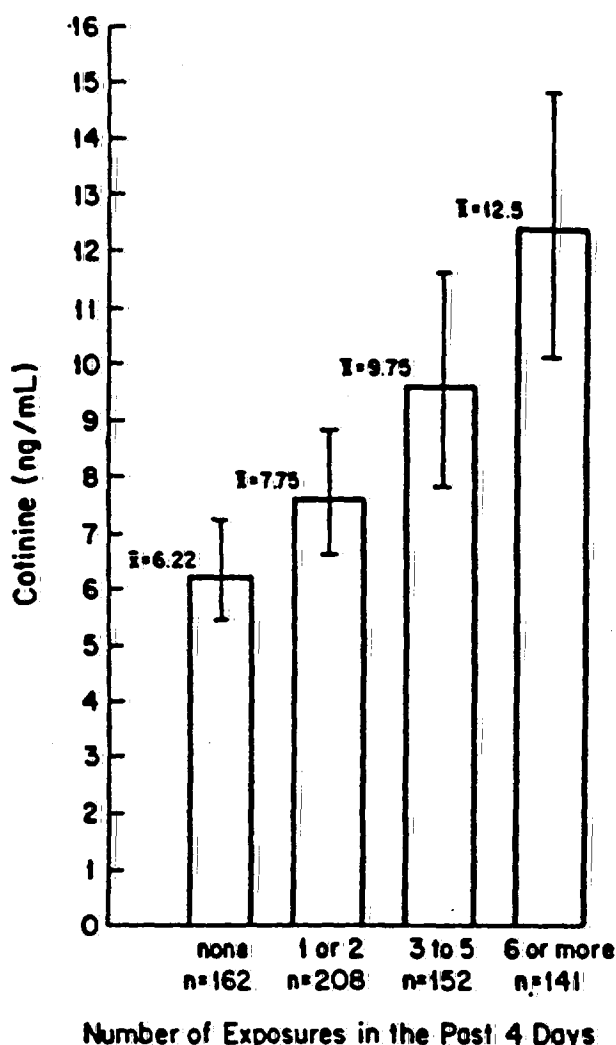


Fig. 1. Urinary cotinine concentrations by number of reported exposures to tobacco smoke in the past 4 d among 663 nonsmokers, Buffalo, New York, 1986.

istics of the exposure location was found to be significantly related to cotinine levels.

A multiple regression analysis was performed to further evaluate the association between the number of exposure reports and cotinine while controlling for the age and sex of the subject and the time of day and month of year when the urine specimens were collected. Overall, this model accounted for 8% of the variability in cotinine levels. Number of exposures and the time of year when urine specimens were collected were the only variables significantly related to cotinine. Each exposure occurrence increased cotinine by 0.58 ng/ml (95% confidence interval: 0.36 ng/ml to 0.80 ng/ml).

Cotinine levels were significantly higher in subjects interviewed during cold weather months (November to April), in subjects who lived with smokers, and in those who reported that most or all of their friends and family whom they see regularly smoke. Cotinine levels also varied significantly by age and race. Younger subjects and nonwhites had higher cotinine levels.

A stepwise multiple regression analysis was performed to evaluate the predictive value of variables found in the bivariate analysis to be associated ($p < .10$) with cotinine. Variables included in the regression model were the subject's age, race, and employment status; the number of smokers who lived with the subject; the number of friends and family members seen regularly who smoked; and the time of the year when the interview was conducted. These six variables accounted for only 7% of the variability in cotinine levels. Three of the six variables were significant contributors to the model. These included, in order of importance, number of friends and family members who smoked; the time of the year when the interview was conducted (cotinine levels higher in subjects interviewed during indoor months, November to April); and the number of smokers who lived with the subject.

The relationship between the characteristics of subjects and reported exposure to passive smoke as measured in the 4-d recall was also assessed. Age, living with a smoker, number of friends and family members who smoked; rules governing smoking at home and in the car, working in a place where smoking is allowed, and time of year when the interview was conducted were all significantly associated with the number of reported exposures. A stepwise multiple regression analysis was performed to evaluate the predictive value of variables found in the bivariate analysis to be associated ($p < .10$) with number of exposures. Variables included in the regression model were the subject's age, employment status, number of smokers who lived with the subject, rules governing smoking at home and in the car, number of friends and family members seen regularly by the subject who smoked, and month of the year when the interview was conducted. These seven variables accounted for 35% of the variance in reported exposure to passive smoke. Six of the seven variables were significant contributors in the model. These included, in order of importance, the number of smokers who lived with the subject; the number of friends and family members seen regularly by the subject who smoked; the subject's employment status (more exposures reported by those currently employed); rules governing smoking in the subject's car;

Table 1.—Relationship between Cotinine (ng/ml) and Measures of Exposure to Passive Smoke from the 4-d Recall

Exposure measures	Correlation with cotinine (ng/ml)	
	Unadjusted	Adjusted for number of exposures
Number of exposures	0.23*	—
Duration of exposure	0.18*	-0.02
Number of smokers	0.19*	0.01
Room size score	0.24*	0.05
Ventilation score	0.25*	0.09†
* $p < .05$.		
† $p < .01$.		

age (more exposures reported by younger subjects); and time of the year when the interview was conducted (more exposures reported by those interviewed during indoor months, November to April). Rules governing smoking at home was correlated with rules about smoking in the car ($r = 0.46$, $p < .01$), which may account for its failure to enter the model as a significant predictor of exposures.

Discussion

Given the self-selected nature of the study population and potentially limited generalizability of results, it is worth noting that the ETS exposure rates reported by study subjects are comparable with exposure rates reported in the literature.¹³ Friedman et al.¹³ found that, among 37 000 nonsmoking members of a prepaid medical plan who were questioned about their exposure to ETS, 63% indicated exposure to tobacco smoke in the previous week. Reported ETS exposure was strongly related to age, with adults in their twenties reporting the highest level of exposure. In this study, three-fourths of non-smokers interviewed reported exposure to tobacco smoke in the 4 d preceding the interview. Similar to the Friedman et al.¹³ finding, ETS exposure was highest among respondents in their twenties and declined steadily with age.

The two most frequently mentioned locations for exposure to passive smoke were at work and at home. Among currently employed subjects, 77% reported being exposed to tobacco smoke at work. Over half of all reported recent exposures occurred in locations where the subject may not have the option to avoid exposure (i.e., at work, in a restaurant, in a public building). This finding suggests that policies regulating smoking in public places could have a substantial impact on reducing a person's exposure to ETS.

The mean urinary cotinine level of 8.84 ng/ml found among nonsmokers in this study is comparable to reports from other studies.^{5,7} By contrast, the mean urinary cotinine level for the 130 smokers tested in this study was 1 254 ng/ml. Among nonsmokers, detectable levels of cotinine were found in the urine of 91% of subjects, including 132 of 162 subjects (81%) who reported no exposure in the 4 d preceding the interview. It is possible that cotinine levels were influenced by exposures that occurred earlier than 4 d reported on in the interview.¹² Also, it is our impression that subjects who are not routinely exposed to ETS may have difficulty recalling instances of exposure.

This study examined several self-reported environmental factors that may influence cotinine levels, including the number of exposures; duration; intensity, i.e., number of smokers; room size; and ventilation characteristics of exposure locations. Consistent with other published reports,⁵⁻⁹ cotinine levels tended to increase with the number of reported exposures to ETS. However, within a given exposure level, there was considerable variability in cotinine values.

Cotinine was chosen as a biological marker of ETS exposure because it is specific to tobacco smoke. However, cotinine levels in body fluids may not only reflect

environmental exposure to tobacco smoke, but also factors that influence uptake and metabolism of nicotine.^{10,12} In controlled laboratory conditions (smoke chambers), it has been shown that duration and intensity of exposure to ETS can affect absorption of nicotine.¹⁰ Results from this study show that accounting for exposure duration and intensity had little influence on cotinine levels once the number of exposures was controlled. Considering the room size and ventilation characteristics of the exposure location also added little to predicting variation in cotinine levels. In questionnaire studies of ETS, it does not appear to be useful to account for characteristics of exposure location, i.e., duration, number of smokers, room size, ventilation factors. Instead, more emphasis should be placed on frequency measures of exposure and the number of smokers among acquaintances.

Findings from this study confirm the results of other investigations, which have found that living with a smoker increases cotinine levels.⁵⁻⁹ However, 84% of subjects who did not live with a smoker had detectable cotinine levels, which underscores the need to consider exposures outside the home. Among the various general exposure measures examined, the best predictor of cotinine was the number of friends and family members seen regularly by the subject who smoked. This measure considers home, workplace, and social exposures to tobacco smoke, and it represents a simple way to evaluate a nonsmoker's usual exposure to ETS.

Cotinine levels were found to vary by month of the year. Subjects who were interviewed during predominantly cold weather months (November to April) reported more frequent exposure to ETS and exhibited significantly higher cotinine levels than subjects interviewed during warm weather months (May to October). The time of the year may not only influence the number of exposures to ETS but also the ventilation characteristics of exposure locations.

Cotinine was assumed to be a valid quantitative measure of ETS exposure in this study. However, there were several potential problems with the cotinine values. Because of the way in which subjects were recruited, it was not possible to fix the day of the week or the time of day when specimens were collected. Jarvis et al.⁶ found that plasma cotinine levels tend to increase in the afternoon. Time of day when the interview was conducted was examined as a potential confounding variable in this study and was found to be unrelated to cotinine levels. Another potential problem with the measurement of cotinine in this study is that values were based on a single random urine specimen. Preferably, cotinine levels should be based on 24-h urine collection to control for variability in the concentration of cotinine between individual urine specimens. In an effort to control for variability in urinary concentrations of cotinine, values were standardized by creatinine excretion, which served as a surrogate measure of urine concentration, and expressed as a cotinine:creatinine ratio. However, parallel analyses done on standardized and unstandardized cotinine values revealed that the correction for creatinine had little effect on the results.

A recent workshop on the measurement of cotinine in nonsmokers recommended, for comparison purposes across studies, that unstandardized values be presented,¹⁴ which is why we have chosen to present our unstandardized cotinine levels.

The relatively modest correlation between reported ETS exposure and urinary cotinine indicates that other factors such as differing metabolic rates and body size may have a confounding effect on the relationship between cotinine levels and questionnaire measures of ETS exposure. In view of this finding, we would recommend against using cotinine levels as a strictly quantitative indicator of ETS. The combination of questionnaire measures of exposure and biologic markers offers perhaps the best approach for accurately assessing recent exposure to ETS.¹⁵

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This work was supported by a grant from the National Cancer Institute (CA40096).

Submitted for publication March 8, 1989; revised; accepted for publication November 8, 1989.

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LETTERS TO THE EDITOR

Lung Cancer and Passive Smoking

Sir - Wald and his colleagues (1990) disagree with Darby and Pike (1988) as to whether the increase in lung cancer risk observed in epidemiological studies in non-smokers in association with exposure to environmental tobacco smoke (ETS) is too large to be satisfactorily explained in terms of their relatively small exposure to tobacco smoke constituents. This is surprising as the discrepancy between the epidemiology and the dosimetry is really very striking.

Table 1 summarises evidence from those 18 epidemiological studies in which risk, relative to a non-ETS exposed never smoker ('Control'), could be estimated both for an ETS exposed never smoker ('Passive') and an ever smoker ('Active'). It also shows the excess risk for the passive group as a percentage of that for the active group. In both sexes this averages 10-20%. Since, as has been widely documented, risk in active smokers is at least linearly related to the amount smoked, one would expect, if there are no major sources of bias, to find that exposure to relevant smoke constituents in the passive group would be at least 10% of that in the active group. However, in fact this is not the case at all. For cotinine, Darby and Pike, citing Jarvis *et al.* (1984) give a value of 0.6-0.8% depending on whether urine, plasma or salivary values are considered, similar to my own estimate of 0.8% (Lee, 1987) based on a nationally representative sample. Wald and his colleagues cite their own data (Wald & Ritchie, 1984) for a somewhat higher figure of 1.5%, but their mean value for exposed non-smokers inappropriately includes some individuals with high cotinine levels that were presumably actually smokers. Not only is there approximately an order of magnitude difference bet-

ween the cotinine results and the epidemiology, but it seems probable that cotinine overestimates the degree of lung exposure from passive relative to active smoking. Whereas in mainstream smoke, nicotine is mainly in the particulate phase and is absorbed through the lungs, nicotine in ETS is mainly in the vapour phase and, being water soluble can be absorbed through the mucous membranes. Arundel *et al.* (1987) have estimated that relative to an average smoker, an average non-smoker retains in the lung 0.02% (males) or 0.01% (females) of the amount of smoking-related particulate matter retained by a smoker. Even multiplying these percentages by two or three to make them applicable to ETS-exposed non-smokers rather than non-smokers in general gives a percentage which is over two orders of magnitude less than the percentage indicated by the epidemiology.

What could be the source of this large discrepancy? Darby and Pike make it clear that it is not duration of exposure, which in any case could well be on average shorter for living with a smoker than for being a smoker. Nor is it because the dosimetry relates to current smoking whereas the epidemiology relates to lifetime smoking as the difference in risk between a current and an ever smoker is much smaller than the size of the discrepancy. Remmer (1987), who also notes the large discrepancy, considers it to be explained by non-smokers being more susceptible to the effects of passive smoking than smokers, because active smoking induces enzymes that protect smokers against these effects, but this explanation seems unattractive and poorly supported by the available evidence. In my view, a much more plausible explanation is that the epidemiological evidence is severely

Table 1 Lung cancer risk in relation to passive and active exposure to cigarette smoke

Sex	Study reference	Relative risk (RR) ^a			% Excess risk passive/active ^b
		Control	Passive	Active	
Female	Iacus & Hirayama (1988)	1.00	2.55	4.25	48%
	Geng <i>et al.</i> (1988)	1.00	2.16	4.18	36%
	Trichopoulos <i>et al.</i> (1983)	1.00	2.08	4.37	32%
	Altaba <i>et al.</i> (1986)	1.00	1.52	3.24	23%
	Brownson <i>et al.</i> (1987)	1.00	1.82	4.75	22%
	Koe <i>et al.</i> (1987)	1.00	1.53	3.56	21%
	Hole <i>et al.</i> (1989)	1.00	1.89	5.43	20%
	Lam & Chung (1988)	1.00	2.01	5.94	20%
	Lam <i>et al.</i> (1987)	1.00	1.65	4.97	16%
	Hirayama (1984)	1.00	1.38	4.12	12%
	Geo <i>et al.</i> (1987)	1.00	1.19	3.15	9%
	Wu <i>et al.</i> (1985)	1.00	1.20	3.31	9%
	Correa <i>et al.</i> (1983)	1.00	2.07	14.10	8%
	Humble <i>et al.</i> (1987)	1.00	2.34	28.53	5%
	Svensson <i>et al.</i> (1989)	1.00	1.26	7.17	4%
	Lee <i>et al.</i> (1986)	1.00	1.03	4.70	1%
	Butler <i>et al.</i> (1984)	1.00	0.80	5.91	-4%
	Chan & Fung (1982)	1.00	0.75	3.07	-12%
	Mean	1.00	1.62	6.38	15%
	Median	1.00	1.60	4.54	16%
Male	Altaba <i>et al.</i> (1986)	1.00	2.10	3.21	30%
	Hirayama (1984)	1.00	2.34	4.39	40%
	Hole <i>et al.</i> (1989)	1.00	3.52	15.88	17%
	Humble <i>et al.</i> (1987)	1.00	4.19	29.36	11%
	Correa <i>et al.</i> (1983)	1.00	1.97	38.15	3%
	Lee <i>et al.</i> (1986)	1.00	1.31	12.82	3%
	Butler <i>et al.</i> (1984)	1.00	0.51	7.63	-8%
	Mean	1.00	2.28	14.58	17%
	Median	1.00	2.10	12.62	11%

^aUnstandardised. Age standardised estimates were only occasionally available and did not differ materially from unstandardised estimates. See text for definition of three categories. ^bCalculated by 100 × (Passive RR - Control RR) / (Active RR - Control RR).

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biased. After all, although the relative risks observed in relation to ETS exposure are large when viewed against the dosimetric evidence, they are small when viewed against the magnitude of effect one can reliably determine by epidemiological methods. A number of sources of potential bias have to be considered - these include publication bias, confounding, inadequate control populations in some studies, and misclassification of active smoking status (Lee, 1989). I have discussed the last of these in detail elsewhere (Lee, 1987; Lee,

1988) and have shown clearly that previous attempts to correct for it (Wald *et al.*, 1986; US National Academy of Science's Committee on Passive smoking, 1986) have been inadequate.

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LETTERS TO THE EDITOR

Response to the Letter from Dr P. Lee

Sir - Lee (1991: volume 63, page 45) claims that there is a discrepancy between the epidemiological and biochemical studies of lung cancer and passive smoking. This conclusion is arrived at by taking the excess risk of lung cancer in passive smokers relative to that in active smokers and comparing it with the difference in biochemical marker levels between active and passive smokers of the same sex and from the same study. This is an invalid comparison.

The epidemiological studies of passive smoking and lung cancer are largely based on women. In virtually all communities of the world women started smoking more recently than men, have smoked less cigarettes per day and have usually smoked cigarettes with a lower tar yield. Their risk of lung cancer from active smoking has correspondingly been lower than for men, but women have been exposed to passive smoke for as long as men have smoked, not for as long as women have smoked. In comparing the epidemiology with the biochemistry it is necessary to relate the risk of passive smoking with the risk of active smoking among men so that the duration of exposure is similar for men and women. We have estimated that (Wald *et al.*, 1986) the relative risk of lung cancer in people who live with smokers compared with people who do not was 1.3 after allowing for misclassification bias. The relative risk of lung cancer in male smokers estimated from the British doctor's study was 14 (Doll & Peto, 1976). The percentage excess risk (passive over active) was therefore about 2% (0.3/13) not 10-20% as Lee suggests. This figure of 2% is similar to the 1.5% difference in urinary cotinine level in non-smokers who lived with smokers compared with non-smokers who lived with non-smokers (Wald & Ritchie, 1984).

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- LEE, in making his dosimetric estimates, cites cotinine data (1987) reported by Jarvis *et al.* (1984) yielding a lower estimate of passive smoke exposure than our own. This is inappropriate, because the study was based on self-defined categories of passive smoking instead of whether the person lived with a smoker, the measure used in the epidemiological studies. Also in an attempt to ensure that subjects were not active smokers, high cotinine levels were censored and some genuine non-smokers who had been heavily exposed to passive smoke may have been excluded thereby underestimating the biochemical measure.
- We do not consider that Lee's analysis casts serious doubt on the evidence on exposure to other people's smoke and lung cancer.

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Passive smoking under controlled conditions

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Summary. Ten healthy subjects were exposed to passive smoking at a high level corresponding to 25-30 ppm CO in the ambient air for 3 h. All subjects were exposed at the same time in a climatic chamber especially designed for exposure experiments. Despite an identical exposure rate considerable interindividual variability of subsequent nicotine and cotinine levels in saliva, plasma and 24-h urine were observed. This variability was more prominent in nicotine than in cotinine levels. The kinetic pattern as reflected by saliva levels for up to 24 h was consistent with previous data found in active smokers. Nicotine levels found in saliva were markedly influenced by repeated sampling. This was not the case for cotinine levels. With regard to laboratory techniques RIA seems to be more sensitive than gaschromatography (GC). The results of this study suggest that measuring cotinine levels in 24-h urine with RIA is presently the most sensitive and reliable criterion for estimating exposure to passive smoking and for validating questionnaires or interviews about short-term exposure to passive smoking.

Key words: Identical exposure - Nicotine - Cotinine - Saliva - Urine - RIA - Gaschromatography

Introduction

A number of studies have been published dealing with the relationship (or lack of one) between passive smoking and increased risk of disease, for example passive smoking and lung cancer [1, 4, 11, 14, 16, 21], respiratory problems in children (e.g. [2, 5, 19]), as well as the changes in lung function in adults (e.g. [3, 15, 23]).

One of the thorniest problems in these studies is that of accurately assessing the exposure to passive smoking.

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In order to clarify some of the current issues in this field an experiment was designed to answer the following questions:

1. What is the kinetic pattern of nicotine and cotinine as measured in saliva following controlled exposure to passive smoking?
2. What is the variability within and between individuals after an identical and high exposure to passive smoking (25–30 ppm CO for 3 h)?
3. Does the collection procedure for saliva influence the results?
4. To what extent are the results of GC and of RIA the same and which of these two methods commonly used is more appropriate for determining nicotine and cotinine levels at low concentrations?
5. What is presently the most appropriate parameter for an objective assessment of exposure to passive smoking and for validating an interview about exposure to passive smoking during the preceding 24 h?

Methods

Subjects

Ten healthy volunteers between the ages of 18 and 65 (5 men and 5 women) took part in the experiment. They were all non-smokers who did not have a smoker in their household.

Exposure

A single exposure session of 3 h was conducted in a 60 m³ climatic chamber especially designed for exposure experiments. The conditions of exposure were controlled so that a high and constant level of tobacco smoke was present in the room.

A smoking machine was used to generate the smoke-polluted atmosphere. It was set so as to draw 15 s followed by a 15 s pause. The mainstream smoke produced during the drawing phase of the cycle (89 ml of smoke per second) was routed outside of the climatic room so that only side-stream smoke polluted the room atmosphere. A total of 24 packs of cigarettes were consumed during the 3-h exposure period.

A continuous reading CO measuring device (Comovarn 100 C, produced by Draeger, Lübeck, FRG) was used to monitor the level of air pollution in the room. A level of CO ranging between 25 and 30 ppm was maintained for the majority of the 3-h exposure interval

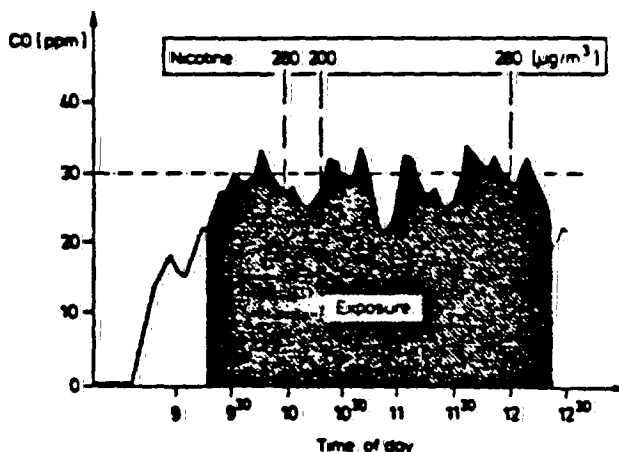


Fig. 1. CO and nicotine concentrations in the air during exposure interval

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(Fig. 1). As a check for the validity of CO as a tracer substance for the level of pollution, air in the room was sampled at 3 points in the time. A measured volume (at least 10 l) was drawn through a tube containing a filter. The mass of captured nicotine was subsequently determined using gaschromatographic techniques. The concentration of nicotine obtained from these samples is also indicated in Fig. 1 and shows that a high and relatively stable level of nicotine pollution was reached and maintained in the room during the experiment. Ventilation was set at 20% fresh air per time unit. Due to the high concentration of side-stream smoke all subjects had to wear goggles throughout the exposure phase of the experiment in order to avoid drop-outs because of unbearable eye irritation.

In order to assure that all individuals were presented with the same level of pollution they were all exposed simultaneously in a single session in the same climatic chamber. In addition, sitting positions were randomly changed at half hourly intervals.

Sample collection

Special attention was paid to the saliva collection procedure. Drinking straws were used together with especially cleaned plastic test tubes and stoppers. Collecting through straws proved to be convenient for the subjects and had the added benefit of limiting the contamination of the sample from nicotine that might be present on the lips. All subjects were advised to let their saliva freely flow through the straw into the test tube until a preset mark (equivalent to 3 ml) was reached. All specimens were deep frozen at -70 centigrade immediately after the collection.

Saliva samples were collected immediately prior to and after exposure and thereafter at three hourly intervals for 12 h. A last sample was taken on the next morning, 21 h after the end of the exposure interval. Twenty-four-hour urine was collected starting immediately before the exposure interval.

Immediately after the end of the exposure interval a sub-experiment was undertaken in which the impact of the saliva collection procedure on measured concentrations of nicotine and cotinine was systematically manipulated. Nine subjects were randomly allocated to three groups. Group I (controls) gave three saliva samples in rapid succession without stimulation. The other two groups gave a preliminary sample in the normal way but their second and third samples were produced under two stimulated conditions: rinsing their mouths with water and lemon juice in a reversed order (Group II: water then lemon, Group III: lemon then water).

Laboratory techniques

Nicotine and cotinine were quantified by radio immuno assay [17]. Selected specimens were reanalyzed by gaschromatography [7] so that a comparison between the two analytical methods could be conducted.

Results

1. Saliva concentrations

At the beginning of the experiment low though measurable baseline levels were observed for nicotine and cotinine in a majority of the subjects (Figs. 2, 3). At the end of the 3-h exposure interval high nicotine levels (median: 89.7 ng/ml; range: 24.7–186.5 ng/ml) were measured. They were followed by a quick drop during the next 3 h until almost baseline levels were reached again. Cotinine, showing a less marked increase, reached its maximum 3 h after the end of the exposure (median: 19.5 ng/ml; range: 14–29 ng/ml) followed by a slow decline. All individual levels of nicotine and cotinine in saliva are also given in Figs. 2 and 3 respectively. Despite substantial variability in the nicotine values

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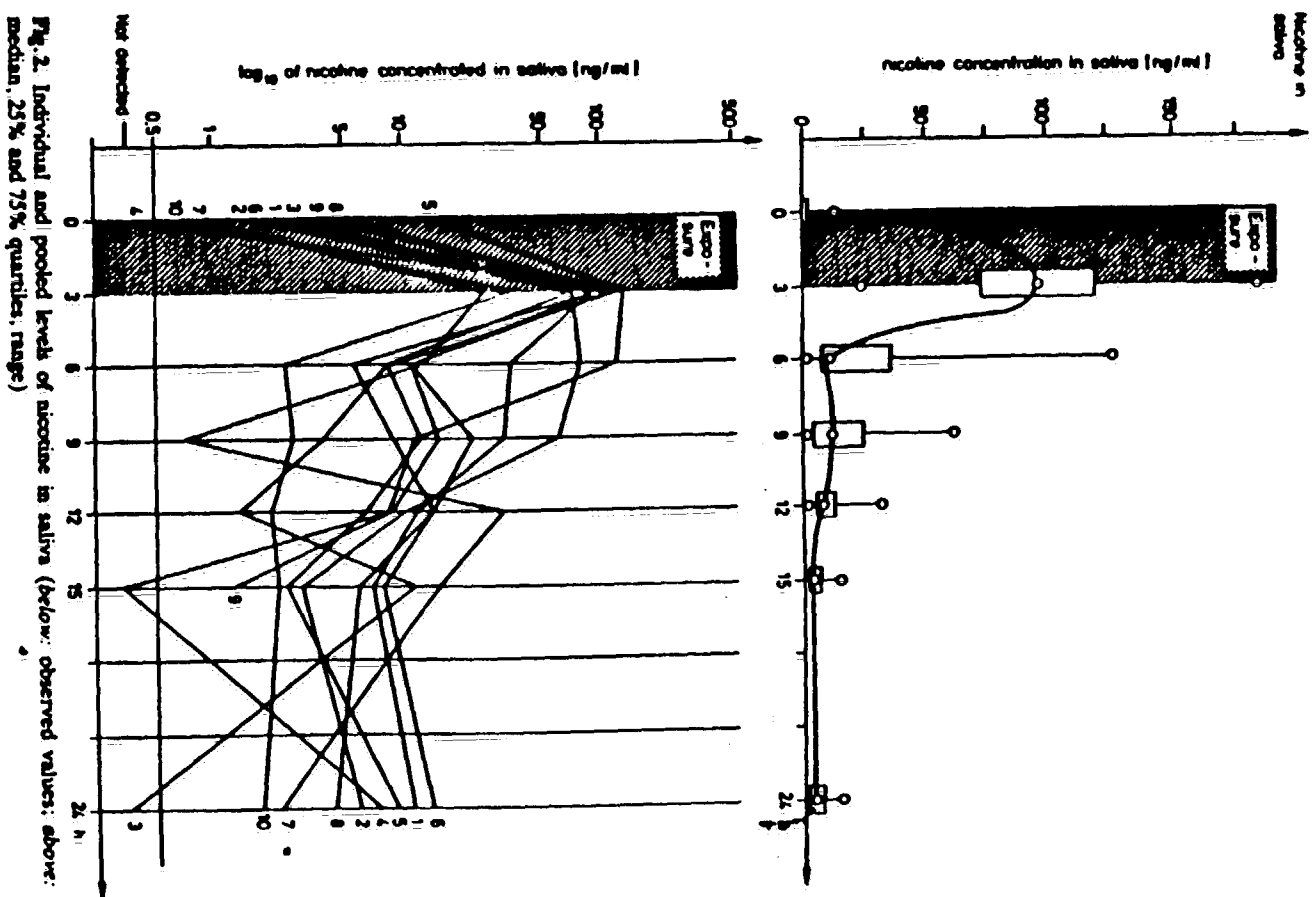


Fig. 2. Individual and pooled levels of nicotine in saliva (below: observed values; above: median, 25% and 75% quartiles, range)

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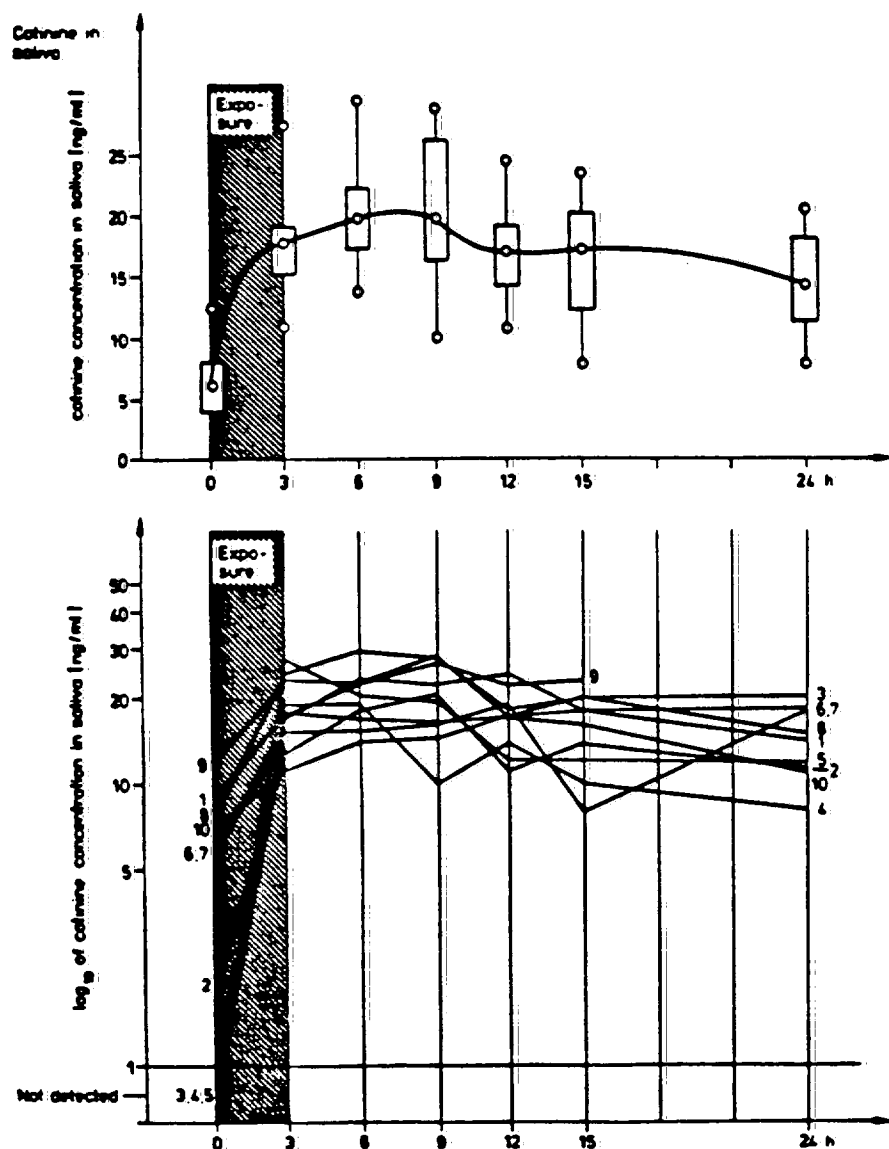


Fig.3. Individual and pooled levels of cotinine in saliva (below: observed values, above: median, 25% and 75% quartiles, range).

the overall trend is consistent with a short half life (30 to 110 min). Similarly the cotinine values are consistent with a substantially longer half life (19 to 40 h). The observed variability in the two substances is different. Whereas nicotine maintains a variability of roughly two orders of magnitude throughout the observational period cotinine values were found to vary substantially less (roughly one half an order of magnitude).

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2. Comparison of analytical methods

For comparing laboratory methods matched samples of saliva, plasma and 24-h urine were analyzed by RIA and gaschromatography (Table 1). The time of sampling was selected in a way that low as well as high concentrations could be compared between the two methods.

Table 1. Comparison of laboratory methods (RIA vs. GC). Saliva and plasma levels are given in ng/ml, urine levels in $\mu\text{g}/24\text{ h}$. Only those samples are included which had detectable levels by both methods. Pre-exposure levels for cotinine in saliva are omitted because only one sample fulfilled this criterion

Sub-stance	Source	Time of collection (hours)	n	$\bar{x} \pm \text{S.D.}$		p (sign test)	r
				RIA	Gaschromatography		
Nicotine	Saliva (ng/ml)	0	8	3.81 ± 4.06	3.87 ± 1.89	1.00	0.78
		6	10	30.3 ± 40.57	37.6 ± 52.95	0.51	0.85
	Plasma (ng/ml)	3	5	3.54 ± 1.58	5.00 ± 1.68	0.37	0.22
	Urine (ng/ml)	Continuously (24 h)	10	134.41 ± 95.84	105.3 ± 88.4	0.04	0.93
Cotinine	Saliva (ng/ml)	6	8	19.7 ± 4.58	18.72 ± 6.25	1.00	-0.43
	Plasma (ng/ml)	3	9	9.00 ± 4.88	8.88 ± 5.31	0.73	0.71
	Urine (ng/ml)	Continuously (24 h)	10	110.21 ± 54.75	82.83 ± 47.3	0.02	0.93

Interindividual variance is considerable. However, neither saliva nor plasma levels show statistically significant differences between the two laboratories. The correlation between the laboratories varies between $r = 0.22$ (plasma nicotine) and $r = 0.93$ (nicotine in 24-h urine).

The analysis of the 24-h urine data showed different results. The means found by RIA are approximately 30% higher than those of gaschromatography ($p < 0.05$). This holds for nicotine as well as for cotinine. There is a high correlation between the two methods for both variables ($r = 0.93$, $p < 0.05$).

In order to compare the sensitivity of the two methods, values for saliva and plasma levels were classified as detectable or not detectable (Table 2). For plasma, the samples collected immediately before and after exposure were pooled. Despite the small number of observations there is a consistent pattern indicating that RIA may be more sensitive than GC. Altogether there were 18 samples with levels detectable by RIA but not detectable by GC. The opposite was found for two samples only. The high incidence of samples without concentrations detectable by either method was not surprising since two thirds of these samples represent baseline levels.

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Table 2. Comparison of sensitivity between RIA and GC. Saliva: baseline levels. Plasma: pooled results from samples collected immediately before and after exposure

			Saliva Detectable by GC		Plasma Detectable by GC	
			no	yes	no	yes
Nicotine	Detectable by RIA	no	0	1	10	0
		yes	1	8	5	5
Cotinine	Detectable by RIA	no	3	0	4	1
		yes	6	1	6	9

3. Influence of saliva collection procedures

The design randomized blocks repeated measurements is illustrated in Fig. 4. Basically, there are three questions that can be investigated by analyzing this complex design:

1. Are the groups comparable, i.e. has the randomization procedure worked? This is answered by comparing sample 1 between the groups.
2. Does repeated sampling itself influence the nicotine and cotinine levels found in saliva after exposure to passive smoking? For this questions, all three samples are compared within group I.
3. How is the concentration of the two compounds affected by saliva secretion stimulation?

		Sample		
		I	II	III
Group	I (n=3)	no stimulation	no stimulation	no stimulation
	II (n=3)	no stimulation	lemon - juice	water
	III (n=3)	no stimulation	water	lemon - juice

Fig. 4. Experimental design for investigating the influence of collecting procedures on saliva levels

Table 3 summarizes the results. Comparing sample 1 between the three groups, very little variation can be discovered between the groups with regard to cotinine (ANOVA: $p = 0.58$), but there appears to be some inhomogeneity regarding nicotine: the concentration of groups I and III is twice as high as that of the one in group II. ANOVA results ($p = 0.16$) suggest, however, that this difference may be accidental.

Group I produced three saliva samples without additional stimulation. The average observed levels for these samples vary substantially for nicotine. The dramatic drop (approximately 50% between sample 1 and 2) is statistically

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Table 3. Nicotine and cotinine levels (ng/ml) in saliva under stimulated and unstimulated secretion conditions

		Sample		
		1	2	3
Nicotine	Group I	98.0 \pm 24.6	59.5 \pm 41.5	48.1 \pm 33.0
	Group II	52.4 \pm 37.0	17.9 \pm 13.6	26.2 \pm 24.3
	Group III	106.0 \pm 32.4	43.3 \pm 28.4	16.9 \pm 5.1
Cotinine	Group I	16.7 \pm 1.5	15.7 \pm 6.1	21.7 \pm 4.0
	Group II	15.3 \pm 3.8	14.0 \pm 1.0	15.3 \pm 2.5
	Group III	18.3 \pm 5.0	17.7 \pm 5.7	15.3 \pm 2.5

significant (ANOVA: $p < 0.05$). This was not the case for cotinine (ANOVA: $p = 0.13$).

For answering the third question the differences in concentration between the first two samples were compared between the three groups. These differences were used not only to adjust for potential inhomogeneities of sample 1 between the groups, but also to remove the repeated sampling effect demonstrated above. ANOVA results (nicotine: $p = 0.29$; cotinine: $p = 0.98$) suggest that stimulating the secretion of saliva does not noticeably influence the concentration of nicotine or cotinine in a second sample.

Discussion

A number of studies have been published which approach the problem of assessing exposure to passive inhalation in one form or another. An early study by Feyerabend et al. [8] is typical in its design and can serve as a model for most of the studies that have been published to date. Using questionnaire techniques non-smokers were classified as to their exposure to tobacco smoke through passive smoking. Subsequently, a particular body fluid (in some studies: expired air) was sampled and analyzed with regard to a particular objective tracer substance. Of interest is the extent to which the exposure classification effectively explains the variation observed between individuals.

In Table 4 we summarize a review of seven such studies. These studies are heterogeneous with regard to the population examined (children, adults, only women), the tracer substance (CO, COHb, nicotine, cotinine, thiocyanate, etc.), body fluid (expired air, urine, blood, and saliva), and laboratory technique. One thing, however, which all of these studies have in common is a large range of observed values (from one to two orders of magnitude depending upon study and tracer substance). In trying to determine the effectiveness of a classification instrument this variation leads to interpretation difficulties. This stems from the fact that subjective and objective assessment of exposure are completely confounded with actual exposure differences as well as individual kinetic differences between individuals. Hence, when one observes a substantial

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Table 4. Summaries of six studies reported in the literature

	Exposure	Measurement of exposure		Lab method	Results
		subjective	chemical		
Feyerabend et al. [8] Nicotine concentrations in urine and saliva of smokers and non-smokers	Work environment	Questionnaire: exposed yes/no	Nicotine levels in urine and saliva	GC	1. Significant correlation between salivary and urinary concentrations ($r = 0.84$) 2. Exposed non-smokers had significantly higher nicotine concentrations than non-exposed subjects
Follart et al. [9] Passive absorption of nicotine in airline flight attendants	Work environment	Questionnaire	1. CO-Hb 2. Nicotine in blood and urine	GC	1. No differences between CO-Hb levels before and after flight 2. Significantly increased blood nicotine concentrations in 5 of 6 subjects 3. Low but measurable nicotine excretion in urine
Friedman et al. [10] Prevalence and correlates of passive smoking	Environment	Questionnaire: exposed hours per week	1. CO in expired air 2. SNC in serum	Not specified	Low correlations between exposed hours per week and chemical parameters ($r = 0.01 - r = 0.15$)
Greenberg et al. [12] Measuring the exposure of infants to tobacco smoke	Mother's smoking	Number of cigarettes smoked by mother	Nicotine and cotinine in saliva and urine	RIA	Significant differences between exposed and unexposed group for all parameters measured
Matsukura et al. [18] Effects of environmental tobacco smoke on urinary cotinine excretion in non-smokers	Work environment	Questionnaire: situational exposure	Cotinine	RIA	Dose response relationship between no. of cigarettes smoked by spouse and levels in urine
Russell et al. [20] Absorption by non-smokers of carbon monoxide from room air polluted by tobacco smoke	Climatized room (CO: 38 ppm for 78 min)	—	CO-Hb	Co-oxi-meter	Significant increase of CO-Hb (1.6% to 2.6%)
Wald et al. [22] Urinary cotinine as marker of breathing other people's tobacco smoke	Environment	Questionnaire: hours per week	Urinary cotinine	RIA	Significant relationship between exposed hours per week and cotinine levels

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variance in observed objective measures it is not clear to what extent this has resulted from true differences in exposure, interindividual kinetic differences, and outright misclassification errors.

The purpose of the experiment reported here was to determine the variability of nicotine and cotinine levels within and between individuals. Great effort was taken to make sure that all subjects had an identical level of exposure. Studies of this type are a necessary step towards accurately assessing exposure in epidemiological studies (e.g. with regard to investigating the relationship between passive smoking and lung cancer). In case-control studies the exposure to passive inhalation must be estimated for (life-)long periods in the past. This is only possible by using questionnaires or interviews. There is a need for validating such a questionnaire before trusting the data. We propose the following procedure:

1. Using equally structured questionnaires, scorable data can be collected from a person regarding the preceding 24 h (in hourly intervals) as well as the total previous life (in yearly intervals).
2. Selecting an appropriate indicator substance, a correlation between the results of the 24-h interview and levels of indicator substance found in body fluids (saliva, plasma, urine) can be determined. If it is high, the method is validated.
3. Having validated the 24-h period it is a matter of believing in analogies whether or not one also trusts a similarly structured life-long history.

In that context, the experiment reported in this paper aims at an approach to validating the results of short-term questionnaires about passive smoking so that future epidemiological studies can be planned more efficiently. The results show that nicotine and cotinine levels are best measured using RIA. The superior sensitivity of RIA is essential since passive smoking under real life conditions leads to very low levels in body fluids.

Combining the work of other investigators with that reported here leads to the following summary with regard to the most appropriate tracer substance. Levels of expired carbon monoxide and/or carboxyhemoglobin in blood are not appropriate since a variety of alternative sources for CO exist (lack of specificity). Similarly, thiocyanate has been shown to be inferior to cotinine both because it is influenced by diet and because it is relatively insensitive at low exposure levels [13]. Nicotine and cotinine can both be measured in blood, urine or saliva. Blood and urine are substantially more difficult to obtain under field conditions than is saliva. In addition, as this study shows, blood levels are very low even after heavy exposure. Both urine and saliva have the potential drawback of being situationally dependent. Nicotine excretion by urine may be related to its pH [6]. Saliva concentrations of nicotine can be influenced by the conditions under which the sampling is conducted (repeated sampling). For all of these reasons nicotine's use as a tracer substance is inadvisable. Cotinine, on the other hand, appears to be well suited for the task.

The body fluid of choice seems to be urine. Although measurable levels of cotinine were observed in this experiment both in saliva and urine, it must be considered that our experiment was carried out under extreme exposure conditions which will hardly ever be reached in real life situations. Our results make

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it seem unlikely that cotinine levels measured in saliva are sensitive enough for validating purposes under real life conditions. The cotinine levels obtained from 24 h urine suggest that an exposure at one tenth of the level used in this experiment would presumably still lead to detectable levels in 24-h urine. Of course, urine samples collected for a shorter period of time also contain measurable cotinine concentrations which, when related to urinary creatinine concentration, can yield meaningful results.

The methodological tools are now available to validate interviews or questionnaires on short-term exposure. Such a validation strategy as outlined in this paper could lead to methods of assessing the exposure to passive smoking which increase the chances for valid epidemiological studies on potential hazards of passive smoking.

Acknowledgements. We wish to thank Prof. Dr. med. K. Überla (Institut für Medizinische Informationsverarbeitung, Statistik und Biomathematik der Ludwig-Maximilian-Universität, München) for his valuable methodological advice. We are indebted to Dr. Nancy Haley, American Health Foundation, Naylor Dana Institute, Valhalla, NY 10595, and her colleagues who gave valuable methodological advice.

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Received October 16, 1984 / Accepted March 3, 1985

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TOXLET 1727

MEASURING PROBLEMS IN ESTIMATING THE EXPOSURE TO PASSIVE SMOKING USING THE EXCRETION OF COTININE*

(Passive smoking; urinary cotinine; controlled low exposure levels; inter-intra-laboratory variation; reliability; measuring problems; fractionated sampling)

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(Received October 7th, 1986)

(Accepted October 13th, 1986)

SUMMARY

Quality control studies on cotinine measurements following low level environmental tobacco smoke (ETS) exposure are rare. The exposure to ETS was controlled and systematically changed in a series of experiments in a climatic chamber. Healthy nonsmoking volunteers were exposed to ETS simultaneously. The duration and level of exposure varied using high (8, 17 and 25 ppm CO), and low (2 and 5 ppm CO) exposure levels. The variation between radioimmunoassay (RIA) and gas chromatography (GC) was high as was the variation between the results of RIA laboratories. There was also a high within-laboratory variation. A 1:10 dilution seems to be preferable over a 1:3 dilution. Freezing the urine samples immediately after collection led to the detection of higher cotinine values than freezing the samples 24 h after collection. Highly reliable data for cotinine were obtained when the urine samples were kept frozen immediately after collection and fractionated sampling over 48-72 h was used. Our data show that estimating low level ETS exposure by measuring urinary cotinine is highly susceptible to uncontrolled variation and errors. Sufficiently reliable estimates of low-level ETS exposure can be made only when fractionated sampling over 48-72 h is used and when the urine samples are kept frozen just after collection.

INTRODUCTION

Cotinine concentrations in urine, plasma, and saliva have been used widely as indicators of recent exposure to ETS. As a metabolite of nicotine cotinine is highly

*Presented at the International Experimental Toxicology Symposium on Passive Smoking, October 23-25, 1986, Essen (F.R.G.)

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Abbreviations: ETS, environmental tobacco smoke; GC, gas chromatography; RIA, radioimmunoassay.

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specific for tobacco smoke. It has been shown that there is a close dose-response relationship between the duration and the level of exposure on one hand, and the urinary cotinine excretion on the other [1-5]. However, the absolute concentrations to be measured can be very low. Following low level of ETS exposure they approach the detection threshold of available laboratory methods and they also show considerable variation. Up to now, with the exception of the paper of Biber et al. [6], we are unaware of studies on quality control regarding the measurement of cotinine following low-level ETS exposure.

A series of experiments in healthy volunteers was carried out in a climatic chamber under strictly controlled conditions. We established a dose-response relationship between experimentally controlled exposure levels and nicotine and cotinine measured in plasma, saliva, and urine. Detailed results have been or will be published elsewhere [2, 14]. In this paper, we report on the results of repeated measurements and the sources of variation which were investigated.

MATERIALS AND METHODS

The exposure sessions lasting from one to six hours took place in a 60 m³ climatic chamber especially designed for exposure experiments. The conditions of ETS exposure were controlled via a continuous-reading CO-measuring device (Comovarn 100 C, produced by Dräger, Lübeck, F.R.G.). A smoking machine was used to generate the ETS atmosphere according to preset levels ranging from 2 to 30 ppm CO. The mainstream smoke produced was routed outside of the climatised room so that only sidestream smoke polluted the atmosphere. As a check for the validity of CO as a tracer substance for the level of pollution, air samples were taken at various points in time. The mass of captured nicotine was subsequently determined by GC. To ensure that all individuals were subjected to the same level of pollution, for each experiment, they were all exposed simultaneously in a single session in the climatic chamber. In addition, sitting positions were randomly changed at 30-min intervals. Nicotine and cotinine in plasma, saliva and urine were quantified by RIA developed by LANGONE [7] with a modification described by Haley [8]. In this paper, only cotinine values will be discussed.

A series of three experiments was conducted. During the first experiment 10 healthy volunteers were exposed to ETS (25-30 ppm CO) for 3 h [2]. This high exposure level was chosen in an effort to establish some preliminary findings:

- the kinetics of nicotine and cotinine;
- the body fluid most suitable for future measurements;
- a methodological comparison of 2 laboratory techniques (RIA and GC).

In a second experiment 2 × 5 healthy volunteers were exposed to ETS corresponding to 8 and 17 ppm CO. The subjects were randomly allocated to 1 or 2 h of exposure. In this experiment we quantified the total urinary cotinine excretion over 72 h as a function of duration and level of exposure. We also compared the cotinine

measurements within one laboratory as well as between two laboratories [14].

In our third experiment we exposed 9 healthy volunteers over 2 and 6 h at an exposure level corresponding to 2 and 5 ppm CO. These exposure levels reflect realistic conditions. They can and do occur in a variety of settings, e.g., at work, with other people smoking in a small and poorly ventilated office [9]. The cumulative urinary cotinine excretion was quantified over 48 h. We examined the impact of interlaboratory variability on the resulting low-level urinary cotinine concentrations. We investigated the variability of the laboratory methods at the resulting low urinary cotinine levels. We also checked on possibilities to increase the sensitivity of the radio immuno assay method by reducing the dilution of the samples from 1:10 down to 1:3 [14]. Dilution of the original samples is commonly used in RIA techniques to reduce cross reactions with other substances.

RESULTS

Variation between RIA and GC

During our first experiment (25-30 ppm CO over 3 h) RIA was compared to GC (Table I). In saliva and plasma no statistically significant differences between the two methods were found, either for nicotine or for cotinine. The correlation between the two methods was relatively weak with correlation coefficients ranging from 0.43 to 0.85.

In 24-h urine the correlation was much higher ($r = 0.93$) but there were statistically significant differences between the distributions of values measured by RIA vs. GC. The means found by RIA were approx. 30% higher than those of GC. This could indicate either a higher sensitivity or a lower specificity of RIA.

To compare the sensitivity of the two methods, values for saliva and plasma levels were classified as detectable or not detectable (Table II). For plasma, the samples

TABLE I
COMPARISON BETWEEN RADIOIMMUNO ASSAY AND GASCHROMATOGRAPHY FOR MEASURING NICOTINE AND COTININE CONCENTRATIONS IN PLASMA, SALIVA AND URINE

Parameter	Source	n	P (sign test)	r
Nicotine	Saliva	8-10	0.51-1.00	0.78-0.85
		9	0.37	0.22
		10	0.04	0.93
Cotinine	Saliva	8	1.00	-0.43
		9	0.73	0.71
		10	0.02	0.93

Statistical analysis was conducted using the sign test.
The linear correlation coefficient r was also calculated (Study I).

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TABLE II
SENSITIVITY OF RADIOIMMUNO ASSAY (RIA) AND GASCHROMATOGRAPHY (GC)

			Saliva Detectable by GC		Plasma Detectable by GC	
			No	Yes	No	Yes
Nicotine	Detectable by RIA	No	0	1	10	0
		Yes	1	8	5	5
Cotinine	Detectable by RIA	No	3	0	4	1
		Yes	6	1	6	9

Only baseline samples or samples with assumed low concentrations of nicotine and cotinine were selected for this part of the analysis. The concentrations measured were dichotomized (detectable/not detectable) (Study I).

collected immediately before and after exposure were pooled. Despite the small number of observations there is a consistent pattern indicating that RIA may be more sensitive (or less specific) than GC. Altogether there were 18 samples with levels detectable by RIA but not detectable by GC. In only 2 samples was the pattern reversed. The high incidence of samples without concentrations detectable by either method was not surprising since two thirds of these samples represent baseline levels.

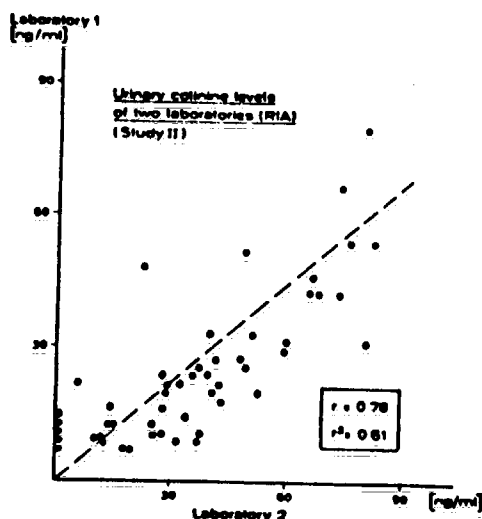


Fig. 1. Variation between laboratories, RIA method according to Langone [7] and Haley [8].

We found that the highest levels were measured in 24-h urine which integrates the excretion over prolonged periods of time. This allows a better estimate of nicotine uptake than levels in plasma and/or saliva; both of which reflect only a point estimate of nicotine and cotinine.

Variation between laboratories

During our second study (8 and 17 ppm CO over 1 and 2 h) we compared urine cotinine concentrations found by two different laboratories both using a comparable RIA method based upon the Langone procedure (Fig. 1). The linear correlation between the values measured by the two institutions is only 0.78. Whether a non-linear model is more appropriate for the data is unclear. Laboratory 1 shows some tendency to systematically measure lower levels of cotinine. But this lab may also have a slightly more sensitive assay at very low levels. There were 5 samples below detection level in laboratory 2, but in laboratory 1 levels between 6 and 14 ng/ml were measured. This indicates that absolute levels found in different studies and measured by different laboratories cannot directly be compared even if they both use the same RIA technique.

Variation by dilution (RIA)

The RIA procedure applied uses a 1:10 dilution of the urine samples. At low or very low levels of cotinine in urine this could mean that the actual concentrations could be diluted below the detection level of the method (approx. 2 ng/ml). In practical terms: minor exposure to ambient smoke may be missed in studies using a urinary cotinine screening for passive smoking of a population. Therefore, we tried

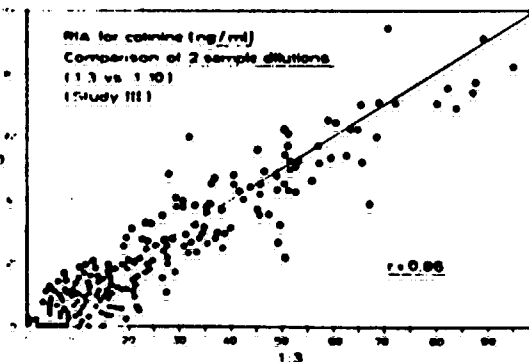


Fig. 2. Variation of urinary cotinine levels between two dilutions of single urine samples (RIA). Values above 100 ng/ml are omitted in this illustration but included for the calculation of the coefficient of correlation.

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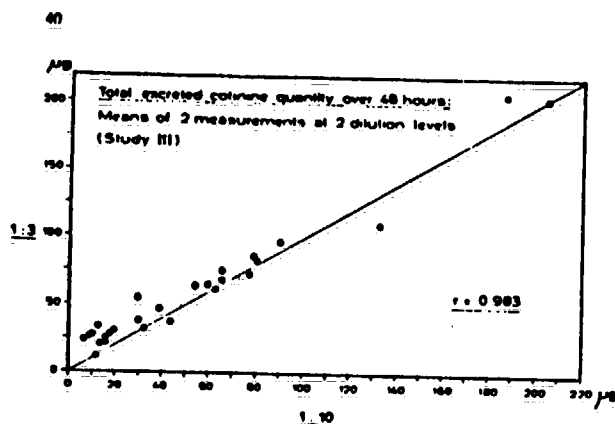


Fig. 3. Variation of two dilutions. Sum of cotinine excretions over 48 h.

to increase the sensitivity of our test during the second study by limiting the dilution to 1:3 (Fig. 2). When comparing the cotinine concentrations in the urine samples of our second study there is an optical tendency to higher levels for the 1:3 dilution. Moreover, considerably more positive samples were obtained at dilution 1:3 than with the higher dilution. Most of these samples without detectable levels at 1:10 were baseline samples obtained before the exposure sessions. Since all participants were known non-smokers who had avoided ETS during the days before the experiment, we conclude that the 1:3 dilution leads to falsely high concentrations caused by more cross reactions. In other terms our findings could mean that an increase in sensitivity of the RIA method is gained at the price of less specificity. Moreover, the cumulated quantity of excreted cotinine over 48 h (Fig. 3) is highly correlated between the two dilutions ($r = 0.983$). There is a tendency to slightly higher recoveries using the 1:3 dilution. Our results indicate that the 1:10 dilution seems to be the better choice.

TABLE III

WITHIN-LABORATORY VARIATION ESTIMATED FROM A SINGLE URINE FRACTION DIVIDED INTO 3 SAMPLES FOR DOUBLE MEASUREMENTS (STUDY II)

Sample	Cotinine in urine (ng/ml)		Mean
	1st measurement	2nd measurement	
1	2.6	4.0	3.3
2	8.4	7.4	7.9
3	4.6	6.9	5.8
4	8.2	3.7	6.0
5	4.3	4.7	4.5
Median	4.6	4.7	5.8

Variation within the laboratory

To obtain information on the variation within the laboratory at low urinary cotinine concentrations we took 5 samples from one urine collection of one person and gave them to one laboratory for double-blind analysis (Table III). The results reported ranged from 2.6 to 8.2 ng/ml (median = 4.65 ng/ml) for the single measurements and from 3.3 to 7.9 ng/ml for the means of each of the 5 pairs. If one divides the total variance of these data into two components – variance between the 5 samples and variance between the 2 measurements of each sample – an analysis of variance reveals that 65% of the total variance can be explained in terms of the variance between the 2 measurements while only 35% is attributable to the variance between the 5 samples. This confirms how important it is to reduce this former variance by using the mean of two measurements. Still, our data indicate that, even when the laboratory techniques are meticulously followed, considerable error variance at low concentrations of cotinine may occur.

This seems to limit the precision with which ETS exposure can be quantified from urinary cotinine levels. This may be true for single measurements but the test-retest reliability of the 2 measurements can be greatly increased by using the total amount of the cotinine mass excreted over the total observation period (e.g., 48 h; Fig. 4). This leads to a correlation coefficient of 0.99 between the two measurements. Since most of the error variance of single measurements is random, pooling double measurements of excreted cotinine mass over an extended period of observation of 48–72 h is the most reliable quantitative indicator of recent ETS exposure.

Variation by freezing the samples

During our second experiment (8, 17 ppm CO over 1 and 2 h) the impact of im-

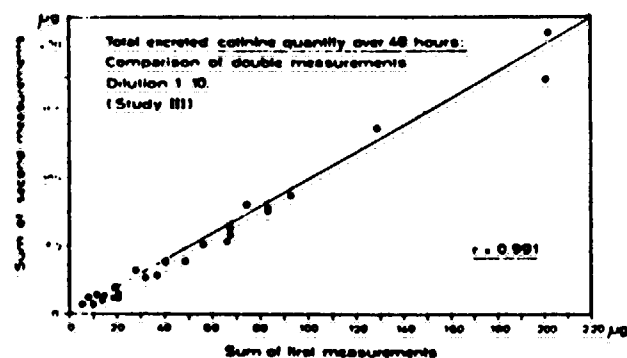


Fig. 4. Variation of urinary cotinine levels within our laboratory. Double measurement of total excreted quantity.

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TABLE IV

MEDIANS OF URINARY COTININE EXCRETION OVER 24 h FOLLOWING POOLED OR FRACTIONED COLLECTION PROCEDURES (STUDY II)

Exposure	Urine collection		Pooled (frozen next day)	
	8 ppm (immediately frozen)	17 ppm (immediately frozen)	8 ppm (frozen next day)	17 ppm (frozen next day)
1 h (n = 3)	1.7 μ g	32.9 μ g	0 μ g	0 μ g
2 h (n = 3)	15 μ g	44.9 μ g	0 μ g	33.7 μ g

mediate freezing of the urine samples obtained in the first 24 h after the beginning of the exposure sessions was analysed (Table IV). Every single urine fraction was collected separately. The volume was recorded; 20 ml were immediately frozen. The rest was pooled in a big sampling bottle and frozen the next day. Consistently higher concentrations were measured when the samples were frozen immediately. This could indicate chemical reactions in the sampling bottle. Another explanation is a dilution of urine quantities containing low but just detectable cotinine levels by earlier urine quantities not yet containing cotinine at sufficient concentrations. Such a mechanism could result in a total concentration of the 24-h urine just below detection level. For these reasons we propose that every single urine fraction should be analysed separately.

Total amount of cotinine excreted over up to 72 h

In studies published up to now cotinine concentrations or cotinine/creatinine con-

TABLE V

MEDIANS OF CUMULATIVE COTININE EXCRETION (μ g) AS A FUNCTION OF OBSERVATION PERIODS (STUDY III)

Exposition	Level	Duration	Cotinine excretion in		Evening-urine + morning-urine	Morning-urine
			72 h	24 h		
8 ppm	1 h		4.3	1.7	1.7	1.2
			(100%)	(39.5%)	(39.5%)	(27.9%)
	2 h		33.4	15.4	8.6	2.6
			(100%)	(46.1%)	(25.7%)	(7.8%)
17 ppm	1 h		64.6	32.9	19.0	12.0
			(100%)	(50.9%)	(29.4%)	(18.5%)
	2 h		79.2	44.9	25.3	12.0
			(100%)	(56.7%)	(31.9%)	(15.2%)

centration ratios were commonly used. These concentrations vary with level and duration of exposure. But being concentrations they cannot be added up over sampling periods. This is one of the reasons why we feel that the total amount of cotinine (volume \times concentration) is a more suitable quantitative indicator of actual exposure. However, the amount of cotinine to be found varies not only with level and duration of exposure but also with the length of time over which the urine is collected (Table V). This can easily be shown in some results from our second study. A single sample, e.g. from the first morning after exposure yields only 8%–28% of the amount of cotinine gained over 72 h. During the first 24 h 40% to 56% can be expected. This shows that the precision of urinary cotinine as a quantitative indicator for the exposure to recent ETS can be increased by prolonged sampling periods of from 48 to 72 h.

DISCUSSION

Several investigators [1–4, 5, 10–13] have shown that measurable concentrations of nicotine and cotinine can be found in plasma, saliva and urine after ETS exposure. Plasma levels in our studies were very low even after extreme exposure conditions. Following low exposure plasma may not be sensitive enough. Moreover, plasma samples are more difficult to obtain in epidemiological studies in a population. Finally, the amount of cotinine found in single plasma samples is confounded by the variable time gap between exposure and sampling. This latter argument holds for saliva levels as well.

The measurement of cotinine excretion in urine is currently the best choice. In our first experiment RIA led to higher cotinine levels and was therefore used for the other two studies. Both methods showed considerable variation from various sources, especially after low ETS exposure. In our investigations we tried to identify some of these sources of variation. There is marked variation between laboratories. From the results presented by Biber et al. at this conference [6] we calculated by analysis of variance that about 60% of the total variance of cotinine levels in urine can be explained by interlaboratory variance. This shows that results for different study groups are not easy to compare, even if apparently identical methods are used. Within laboratory variation is also high but can be controlled experimentally by accumulating the total cotinine mass found during continuous urine sampling over a period of 48 to 72 h after exposure and by double measurements. The specific conditions under which the RIA method is conducted influences the actual levels to be found: less dilution of the original samples increases sensitivity at the cost of specificity at low levels. The most stable indicator of recent low-level ETS exposure is to analyse the total urine excreted over a period of 48 to 72 h using a fractional sampling technique. The total amount of cotinine excreted over this period of time is superior to cotinine concentrations and/or cotinine-creatinine ratios found in single urine samples.

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We have shown that without fractioned sampling over 48-72 h there is substantial variation and we recommend, therefore, that this technique should be used in future studies.

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INDEX

DETERMINATION OF NICOTINE AND COTININE IN HUMAN SERUM AND URINE: AN INTERLABORATORY STUDY*

(Smokers; non-smokers; radioimmunoassay; gas chromatography; interassay variation)

ANTON BIBER^a, GERHARD SCHERER^b, ILE HOEPFNER^c, FRANZ ADLKOEFER^{b,c}, WOLFGANG HELLER^c, JAMES E. HADDOW^d and GEORGE J. KNIGHT^d

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(Received 15 September 1986)

(Accepted 25 September 1986)

SUMMARY

An interlaboratory study aimed at determining nicotine and cotinine in human serum and urine was carried out. 11 laboratories from 6 countries, all experienced in performing nicotine and cotinine determinations in biological fluids by radioimmunoassay (RIA) and/or gas chromatography (GC) were involved. Each of them received 18 serum and 18 urine samples. The specimens were obtained from 8 smokers and 10 non-smokers; 2 samples from non-smokers were spiked with defined amounts of nicotine and cotinine. All the laboratories distinguished perfectly between the smokers and the non-smokers and according to cotinine levels in serum the laboratories ranked the samples with good agreement. There were systematic differences in the absolute values between the laboratories. The ratios of urinary cotinine concentrations between active and passive smokers differed widely from laboratory to laboratory. The reasons for this are not yet known and necessitate further investigation.

*Presented at the International Experimental Toxicology Symposium on Passive Smoking, October 21-25, 1986, Ewen (F.R.G.).

**To whom correspondence and reprint requests should be addressed.

Abbreviations: c.v., coefficients of variation; ETS, environmental tobacco smoke; GC, gas chromatography; RIA, radioimmunoassay.

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The authors are indebted to all the scientists participating in this study. Without their spontaneous cooperation it would not have been possible to carry out this investigation.

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Elsevier

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MATHEMATICAL MODELLING OF NICOTINE AND COTININE AS BIOLOGICAL MARKERS OF ENVIRONMENTAL TOBACCO SMOKE EXPOSURE*

(Physiological pharmacokinetic models; computer simulations)

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(Received 15 September 1986)

(Accepted 23 September 1986)

SUMMARY

Computer software developed in our laboratory (CMATRIX) was used to design a physiological pharmacokinetic model of nicotine absorption, distribution, metabolism and excretion in man. The model accumulates inhalation of nicotine from various environmental settings and physiological conditions in man. It was also used to predict pharmacokinetic behavior of cotinine arising from nicotine metabolism. Model-predicted variations in body-fluid nicotine levels confirm that nicotine is not an acceptable quantitative marker of environmental tobacco smoke (ETS) exposure. Though cotinine provides a more stable pattern, predicted interindividual variation suggests the need for specific strict sampling and monitoring guidelines for cotinine to be a reliable quantitative marker.

INTRODUCTION

Epidemiological and human toxicological studies of ETS require a means for exposure assessment. Emphasis is currently given to nicotine and its major metabolite cotinine as biological markers of ETS exposure. These two compounds have obvious qualitative specificity. This study was directed at establishing criteria necessary for using nicotine or cotinine as a quantitatively reliable ETS marker.

* Presented at the International Experimental Toxicology Symposium on Passive Smoking, October 23-25, 1986, Fosen (F.R.G.).

Abbreviation: AUC, area under the curve; ETS, environmental tobacco smoke.

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Clearance and volume of distribution control the dose-tissue concentration relationship. Between and within subject variations commonly result from variations in these two parameters. For example, consider:

$$Cl_H = Q_H \times E \text{ and } E = Cl_i / (Q_H + Cl_i)$$

where Cl_H is hepatic clearance, Cl_i is intrinsic hepatic clearance, i.e., liver metabolic capacity for the substance; E is hepatic extraction ratio and Q_H is hepatic blood flow. Individual to individual differences in Cl_i and, therefore, E and Cl_H are common for high Cl_i substances. Also, where Cl_i is high, Cl_H becomes more dependent on Q_H . Individual variations in hepatic blood flow (e.g., via postural changes) are well known. Similarly, volume of distribution can vary from individual to individual. A substance should be completely distributed in that volume of distribution for there to be quantitative predictability.

METHODS

Specific points addressed in this study were: (i) sample timing requirements; (ii) nicotine dose vs. measurable cotinine; (iii) kinetics of inhaled nicotine; (iv) the effects of hepatic clearance variations on quantitative predictability.

The predictability question was approached through the use of physiological pharmacokinetic modelling and computer simulation. Bischoff, Dedrick and coworkers [1,2] introduced the use of physiological pharmacokinetic models to represent drug distribution. Flow-limited distribution among tissues can be represented by a series of simultaneous linear and non-linear differential equations based on the Fick principle and numerically solving the equations by reiterative analysis.

Computer simulations were done using CMATRIX [3]. This is a general method developed in our laboratory for designing physiological pharmacokinetic models that is based on mathematical graph theory. Sequential flow from the gut to the liver was accommodated in the model.

Three types of simulation were done. For the first, nicotine was administered intravenously and the disposition of nicotine only followed. For the second, nicotine was administered intravenously, the disposition of nicotine and the metabolite followed. All metabolite was assumed to be cotinine. This is obviously not the case in the real system, but for study purposes and execution it was a useful assumption. The third type of simulation involved exposure to nicotine by inhalation.

Modelling parameters for nicotine were as follows and are presented as tissue, tissue volume (l), blood flow (l/min) and tissue/blood ratios, respectively: blood - 5.4, 6.1 (cardiac output), 1; liver - 1.5, 1.55 (hepatic artery plus portal vein), 5.6; GI tract - 2.5, 1.25, 3.4; kidney - 0.3, 1.25, 3.7; muscle - 34.4, 1.75, 2; fat - 10, 0.2, 0.5; brain - 1.5, 0.80, 3.7; balance of vessel-rich group - 0.65, 0.55, 6. Renal

clearance was established as 0.2 l/min while the hepatic clearance was varied between 0.64 and 1.31 l/min. The inhalation model included the assumption that the blood/gas partition coefficient for nicotine is 100. The only basis for this assumption is the recognition that the coefficient must be very high. There is very little difference in kinetic behavior from one very high coefficient to another, especially for a chemical with a short half-life. For cotinine, the tissue/plasma ratios were set at 1.0 for all tissues; hepatic and renal clearances were 0.055 and 0.011 l/min, respectively.

The basic nicotine and cotinine models were designed to produce data consistent with the human data reported for nicotine and cotinine by Benowitz and coworkers [4,5]. Tissue/plasma ratios for nicotine were adapted from unpublished data from the rabbit and were kindly provided by Dr. Neil Benowitz, University of California at San Francisco.

RESULTS AND DISCUSSION

Certain of the pharmacokinetic data from the simulations are shown in Figs. 1 through 4 and Table I. Simulation No. 1 (Fig. 1) was a simulation of a study reported by Benowitz et al. [4]. The effects of varying nicotine hepatic clearance on the nicotine and cotinine blood levels and on the urinary excretion of both compounds are quite evident. It is hardly surprising that this occurs. But the simulations graphically demonstrate some important points on the use of nicotine or cotinine

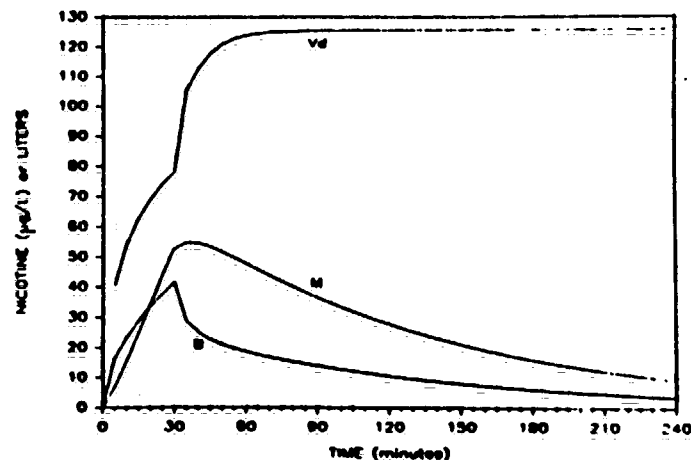


Fig. 1. Simulation No. 1 in Table I. B, blood; M, muscle; Vd, apparent volume of distribution.

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TABLE I
RESULTS OF NICOTINE SIMULATIONS

No.	Route	Dose	Duration	V_d (l/min)	V_d (l)
1	i.v.	2 μ g/kg/min \times 30 min	4 h	1.00	129
2	i.v.	2 μ g/kg/min \times 4 h	4 h	1.00	129
3	i.v.	2 μ g/kg/min \times 4 h	4 h	1.11	136
4	i.v.	2 μ g/kg/min \times 4 h	4 h	0.64	117
5	i.v.	2 μ g/kg/min \times 4 h	24 h	1.11	136
6	i.v.	2 μ g/kg/min \times 4 h	24 h	0.64	117
7	inhal.	1 mg/m ³ \times 4 hr ^a	5 h	1.31	136
8	inhal.	1 mg/m ³ \times 4 hr ^a	5 h	0.64	117
No.	AUC blood ^b mg-min/ml	Peak blood mg/l	Excreted in urine	Metabolized by liver	
2	25.00	107	5.43 mg/4 h	24.49 mg/4 h	
3	26.90	99	4.60 mg/4 h	26.30 mg/4 h	
4	32.65	139	6.56 mg/4 h	26.08 mg/4 h	
5	22.64	99	4.95 mg/24 h	28.40 mg/24 h	
6	271.51 ^c	208	4.04 mg/24 h	15.87 mg/24 h	
7	40.40	139	0.37 mg/24 h	24.90 mg/24 h	
8	231.97 ^c	234	3.45 mg/24 h	13.55 mg/24 h	
7	1.43	7.64	0.38 mg/4 h	1.65 mg/4 h	
8	1.26	10.63	0.49 mg/4 h	1.07 mg/4 h	

^aNephric clearance. Renal clearance was 0.2 l/min for all simulations.

^bIncludes smoking cigarette disposition.

^cVentilation rate, 10 l/min; alveolar volume, 4.5 l.

^dAUC: area-under-curve determinations for duration of simulation except for Nos. 7 and 8, which were determined at 4 h.

^eValues for nicotine are in this row.

as quantitative markers of ETS exposure. Determinations from spot tissue fluid samples are inadequate. For episodic ETS exposure (e.g., workplace) AUC_{0-∞} computed from repeated sampling is necessary to adequately estimate the body burden of nicotine and cotinine from blood. The same holds true for estimations made from saliva.

24-h urine collections are required when urine is used for body-burden estimates. Differences in clearance and volume of distribution can be compensated for by using both blood and urine for measurements. This is especially advantageous when exposure is continuous.

The simulations provided initial information on the relationship between atmospheric concentration and body-fluid and tissue levels of nicotine and its metabolites. Based on a comparison to Simulation Nos. 3 and 4 (Table I). Simulation Nos. 7 and 8 resulted in nicotine intake of 179 mg/kg/min and 164 mg/kg/min, respectively. Physiologically, it is expected that the higher the blood level achieved

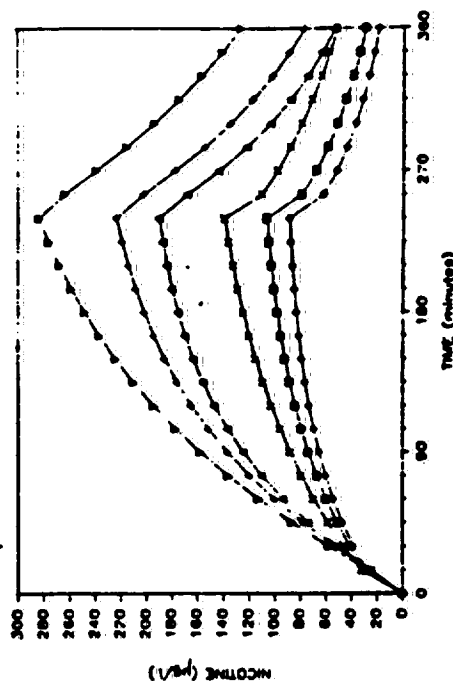


Fig. 2. 1, simulation No. 2, blood; 2, simulation No. 3, blood; 3, simulation No. 4, blood; 4, simulation No. 5, muscle; 5, simulation No. 3, muscle; 6, simulation No. 4, muscle; 7, simulation No. 5, muscle; 8, simulation No. 6, muscle. See Table I for key to simulation numbers.

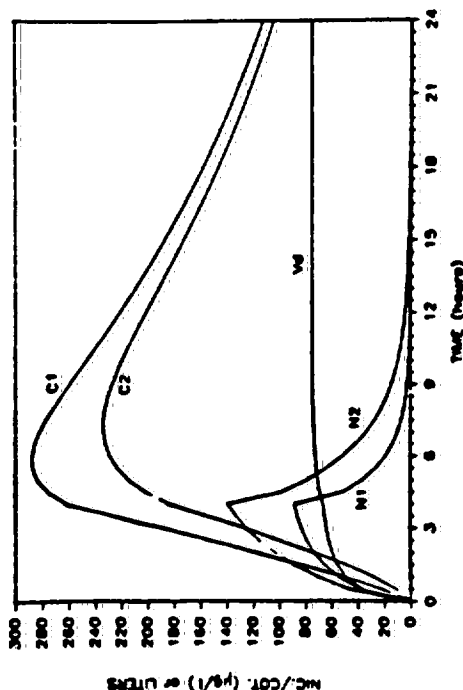


Fig. 3. N1, nicotine in blood, simulation No. 5; N2, nicotine in blood, simulation No. 6; C1, nicotine in blood, simulation No. 5; C2, nicotine in blood, simulation No. 6; Vd, nicotine apparent volume of distribution, simulation Nos. 5 and 6. See Table I for key to simulation numbers.

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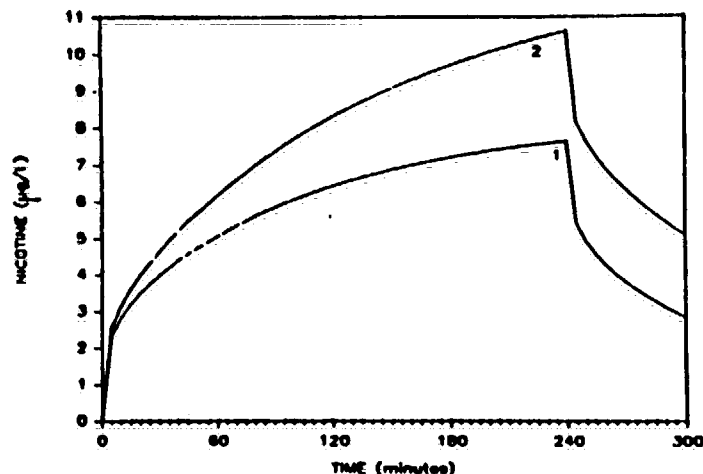


Fig. 4. 1, nicotine in blood, simulation No. 7; 2, nicotine in blood, simulation No. 8.

on inhalation the less the net fractional absorption from subsequent exposure. It remains to be seen experimentally if the blood/gas partition coefficient used for nicotine is appropriate. However, the simulation clearly points out the necessity for having and applying this information.

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ISI: 01713

DEPOSITION RATES OF SIDESTREAM TOBACCO SMOKE PARTICLES IN AN EXPERIMENTAL CHAMBER*

(Indoor pollution; environmental tobacco smoke; smoke measurement; smoke marker; particle residence time; smoke dissipation rate)

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(Received 8 September 1986)

(Revision received 18 September 1986)

(Accepted 25 September 1986)

SUMMARY

The natural dissipation rates of sidestream smoke (SS) particles dispersed in a chamber were studied from the standpoint of a static atmosphere and were expressed as half-lives of residence in the air. The half-lives for particles $<0.3 \mu\text{m}$, $0.3-0.5 \mu\text{m}$ and $0.5-1 \mu\text{m}$ were found to be 25.5, 12.8 and 4.9 h, respectively. Total particulate matter (TPM) decreases by half after 6.2 h. Other data on diluted SS in the indoor air were also reported.

INTRODUCTION

Environmental tobacco smoke (ETS) is part of the more general problem of indoor air pollution [1]. Annoyance and irritation of the mucous membranes of the eyes and the respiratory system following involuntary exposure to ETS were reported [2] as well as heart disease [3] and risk of lung cancer [4]. In a closed space ETS, which was pointed out to be a mixture of SS and exhaled smoke [5], constitutes an important source of pollution.

*Presented at the International Experimental Toxicology Symposium on Passive Smoking, October 23-25, 1986, Essen (F.R.G.).

Abbreviations: ETS, environmental tobacco smoke; RH, relative humidity; SS, sidestream smoke; TPM, total particulate matter.

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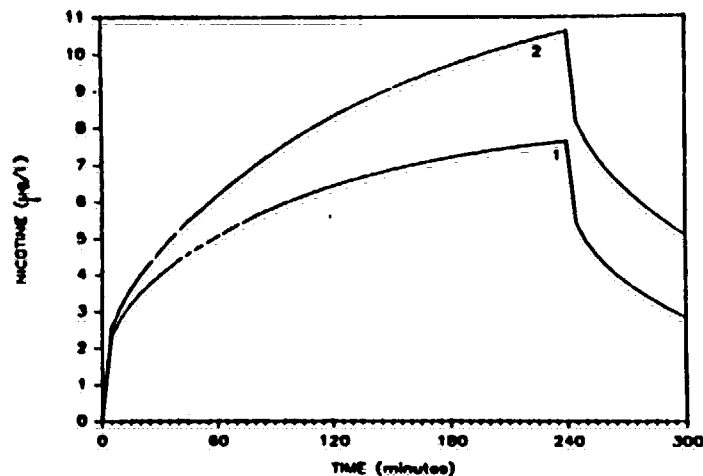


Fig. 4. 1, nicotine in blood, simulation No. 7; 2, nicotine in blood, simulation No. 8.

on inhalation the less the net fractional absorption from subsequent exposure. It remains to be seen experimentally if the blood/gas partition coefficient used for nicotine is appropriate. However, the simulation clearly points out the necessity for having and applying this information.

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ISI: 01713

DEPOSITION RATES OF SIDESTREAM TOBACCO SMOKE PARTICLES IN AN EXPERIMENTAL CHAMBER*

(Indoor pollution; environmental tobacco smoke; smoke measurement; smoke marker; particle residence time; smoke dissipation rate)

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(Received 8 September 1986)

(Revision received 18 September 1986)

(Accepted 25 September 1986)

SUMMARY

The natural dissipation rates of sidestream smoke (SS) particles dispersed in a chamber were studied from the standpoint of a static atmosphere and were expressed as half-lives of residence in the air. The half-lives for particles $<0.3 \mu\text{m}$, $0.3-0.5 \mu\text{m}$ and $0.5-1 \mu\text{m}$ were found to be 25.5, 12.8 and 4.9 h, respectively. Total particulate matter (TPM) decreases by half after 6.2 h. Other data on diluted SS in the indoor air were also reported.

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(2)

Biochemical Archives, Vol. 2, pp. 91-97, 1980
Printed in the U.S.A.

H.B.R. Press

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DIETARY NICOTINE AND ITS SIGNIFICANCE
IN STUDIES ON TOBACCO SMOKING

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Abstract

There is increasing interest in ingestion of nicotine by non-smokers, and the popular assumption is that inhalation of tobacco smoke is the sole source of this alkaloid in body fluids of non-smokers. However, sources other than tobacco (particularly dietary) have been largely overlooked. In the present study, we measured nicotine levels in various solanaceae (tomato, pepper and eggplant) utilizing radioimmunoassay in order to understand the role of these food sources in the ingestion of nicotine. Our findings showed large quantities of nicotine in ripe fruit, and even greater amounts in unripe fruit.

Research on the distribution of nicotine and its metabolites includes not only tobacco smokers who may receive several milligrams of nicotine per day, but also non-smokers who may ingest trace quantities of the alkaloid through air contamination and non-tobacco sources. Comparative studies on the biotransformation of nicotine in man and other animals has led to an ongoing exploration of methods for measurement of nicotine and related compounds. These methods range from early paper chromatographic procedures through gas chromatographic, combined gas chromatographic-mass spectrometric, mass spectrometric, thin-layer, and high-performance chromatographic procedures (1-5). Concurrently, several radioimmunoassays have been developed for nicotine (6-11). A previous review compared some of the advantages and disadvantages associated with the various methods (12).

In recent years, interest in nicotine metabolism has been stimulated by increased consideration of the role of nicotine as a reinforcer in the habitual use of tobacco (13) and by studies on the short half-life of nicotine in human blood plasma (14-16). Further, some nicotine metabolites appear physiologically to oppose or enhance the effects of nicotine (1,17,18). These and other considerations have contributed to the development of effective methods for determining levels of nicotine in biological fluids.

Limitations and advantages of gas chromatography and combined gas chromatography/mass spectrometry and other methods have been previously reviewed (3,12). Because of the specificity and sensitivity of such assays, several researchers have investigated various immunological techniques for the rapid determination of nicotine. Radioimmunoassay (RIA) for nicotine has been developed (9,19), but

0749-3333/86 \$3.00 + .00

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commercial preparations of ^3H - or ^{14}C -labelled nicotine of high specific activity have become increasingly scarce. ^{125}I -labelled nicotine of high specific activity has been prepared (9), but its shelf-life is short and there is considerable lot-to-lot variation.

During the past two decades numerous investigators have attempted to relate the degree of exposure (ie, light puffing vs. deep inhalation vs. 'passive' smoking from air contamination by non-smokers) to smoke from tobacco products, especially cigarettes, to the amount of nicotine found in various tissues and fluids of the body. However, too many individual factors are involved for a precise correlation to be shown between levels of nicotine in blood and the degree to which tobacco smoke has been inhaled.

In order to obtain more accurate values for half-life of nicotine in smokers and non-smokers, we were led to examine various other sources of nicotine, especially dietary sources such as solanaceae (tomato, pepper and eggplant). Early data on the presence of nicotine in tomatoes and eggplants was highlighted at a 1964 symposium (4th Wenner-Gren Symposium, Stockholm). It was pointed out that the wide distribution of nicotine in plant material suggests that both tobacco users and abstainers encounter nicotine as a dietary constituent.

Materials and Methods

Preparation of Immunogens

6-(ϵ -aminocapramido)-DL-nicotine. Isobutylchloroformate (1.32 ml; 10 mmole) was added slowly to a cooled solution of 2.313 gm (10 mmole) of ϵ -BocCaproic acid and 1.4 ml (10 mmole) of triethylamine in 20 ml of dry toluene at -5°C . After 25 minutes at this temperature, 1.25 mmole of 6-amino-DL-nicotine in 5 ml of dry toluene was added. Rapid CO_2 evolution began, and the reaction proceeded overnight at room temperature. Triethylamine hydrochloride was removed by filtration and the toluene was removed under reduced pressure. Ethyl acetate was added to the yellowish oily product, which was washed four times with a solution of 10% sodium bicarbonate saturated in sodium chloride, three times with distilled water, dried over anhydrous sodium sulfate and filtered. The solvent was removed under reduced pressure and the gum-like product was dissolved in ether, dried over anhydrous sodium sulfate, and yielded 1.81 gm (46% yield) of oily product upon removal of the solvent. Electrophoresis ($\text{CH}_3\text{COOH}/\text{C}_2\text{H}_5\text{N}/\text{H}_2\text{O}$ 22.8/32.3/41, pH 5) indicated a trace of 6-amino-DL-nicotine in the product: (ir $^{\text{KCl}}$) 1170 (ν , C-N), 1698, 1706, 1726, (ν , C=O). 3220, 3435 and 3445 cm^{-1} (ν , NH). The 6-(ϵ -aminocapramido)-DL-nicotine was obtained by removal of the Boc-group from 6-(ϵ -BocCapramido)-DL-nicotine using hydrogen chloride in tetrahydrofuran. Chemicals were obtained from Aldrich Chemical Company.

6-(ϵ -aminocapramido)-DL-nicotine-BSA conjugate. A solution of 192 mg of BSA in 3 ml of water was added to a solution of 847 mg of 6-(ϵ -aminocapramido)-DL-nicotine in 10 ml of water which was previously adjusted to pH 9 at room temperature. 1-ethyl-(3,8-dimethylaminopropyl) carbodiimide hydrochloride (650 mg) was added in one portion to the above solution with stirring. The pH of the reaction was maintained at 9 through the first two hours. An additional 650 mg of carbodiimide was added after one hour and the reaction mixture was allowed to stand at

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room temperature for 72 hours. The conjugate was dialyzed for three days in 8 liters of phosphate buffer (0.01M, 7.8 pH) and finally one day in sodium chloride/phosphate buffer (0.01M, 7.8 pH). (The buffer solution was changed twice daily). It was found that the nicotine/BSA ratio was 1:1 based on tracer method calculations.

Administration of Immunogens

A saline suspension of antigen emulsified with an equal volume of complete Freund's adjuvant was injected into 6-lb (average) New Zealand rabbits and into 50-lb (average) goats. The schedule of injection in the different groups was as follows:

Rabbits. 6-(ϵ -aminocapramido)-DL-nicotine-BSA, 2 mg of conjugate/rabbit in 1 ml, in toe pads of all four feet (0.1 ml/site); 2 weeks later each rabbit was injected in both hind legs with 0.5 ml of the emulsion (2 mg conjugate in .5 ml saline and .5 ml of complete Freund's adjuvant). Two weeks later the dose was repeated in both hind legs and harvesting of antibodies was started one week after the third injection.

Goats. 6-(ϵ -aminocapramido)-DL-nicotine-BSA, 1 mg of conjugate/goat in 1 ml, in hind leg; 2 weeks later each goat was given a booster in hind leg with 0.5 ml of the emulsion (1 mg conjugate in .5 ml saline and .5 ml of complete Freund's adjuvant). Two weeks later the dose was repeated in the hind leg and harvesting of antibodies was started one week after the third injection.

Radiolabelled Ligands

The tyrosine methyl ester conjugate of 6-aminonicotine labelled with 125 I, which has been previously described (19), was prepared in the research department of Union Carbide Corp.

Cross Reactivity Studies

Cross reactivity studies were carried out by a method reported earlier (7). Percent cross reaction was calculated by the method of Abraham et al (20). Final antiserum dilutions used were 1:8000 (6-(ϵ -aminocapramido) nicotine-BSA).

Studied Materials

We examined various solanaceae for nicotine content. Most of the fresh vegetables were obtained from the University of Florida Agricultural Research Station, Homestead, FL. The canned products, ripe beefsteak tomatoes, and egg-plant were obtained in local supermarkets. Material was homogenized and the nicotine content was reported as ng/g of wet weight.

Results and Discussion

Cross-reactivity studies. Structure and results of cross-reactivity studies for each of four antibodies are shown in Table 1. Antiserum I showed the highest specificity to (R,S)-6-aminonicotine (146% at 1 hr and 156% at 24 hrs), and during the first hour was more reactive with (S)-nicotine than (R)-nicotine. Slight cross

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TABLE I
Crossreactivity Studies on Antisera to Nicotine Produced in Rabbits
and Goats from Conjugates of BSA and Functionalized Nicotine

Ligand	Relative Crossreactivities (%) after incubation			
	Rabbit*		Goat**	
	1 hr	24 hr	1 hr	24 hr
S-nicotine (VI)	100	100	100	100
R-nicotine (VI)	76	100	107	98
R,S-6-aminonicotine (I)	146	156	119	310
S-Nicotine-N'-oxide (IV)	29	17	3.2	316
S-Nicotine dimethonium iodide (III)	0.1	0.1	0.1	0.1
S-Nicotine isomethonium iodide (XII)	0.1	0.1	0.1	0.1
S-Cotinine (V)	0.1	0.1	0.1	0.1
N-methyl pyrrolidine (VII)	0.1	.0	.06	0.1
S-4-(α -Pyridyl)-4-methyl-amino-butyric acid (VIII)	0.1	0.1	0.1	0.1
S-Nornicotine (IX)	0.8	0.4	0.3	0.6
S-N'-nitrosonor-nicotine (X)	0.1	0.1	0.1	0.1
p-Aminobenzoic acid (XI)	0.1	0.1	0.1	0.1

* diazotized 6-amino-nicotine conjugate
** 6-L-amino-capramide nicotine

In the United States, tomatoes are second only to potatoes in importance as a table vegetable. Green tomatoes are used in relishes and pickle preparations, and peppers are also widely used. Effects of storage in increasing or decreasing nicotine in tobacco have been studied in some detail. However, changes in nicotine levels of foodstuffs during storage are not well understood. In this study we found nicotine in commercially canned tomatoes and tomato paste at levels higher than those reported by us for the whole ripe fruit.

It should be noted that with agricultural products, composition is significantly influenced by strain, soil, fertilizer, weather, degree of ripeness, etc., so that only approximate values can be obtained. However, our studies indicate that large quantities of nicotine can be acquired through diet. The relationship of these findings to human disease associated with nicotine requires further investigation.

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TABLE II
Nicotine Content of Solanaceae Studied

Vegetable	Nicotine*
Beefsteak tomato (ripe)	7.9
Salad tomato (ripe)	9.8
Bell pepper (fresh)	5.7
Mungwar pepper (fresh)	5.7
Florida Dade 79029 tomato (green)	42.8
Florida Dade 79029 tomato (ripe)	4.3
PI 14403 tomato (green)	14.2
PI 14403 tomato (ripe)	5.6
PI esculento 272669 (green)	8.9
PI esculento 272669 (ripe)	5.3
Amazon Columbia (green)	25.5
Amazon Columbia (ripe)	3.3
Eggplant	100.0
Eggplant (boiled)	32.0
Progreso puree salsadi pomidoso (peeled tomatoes)	52.0
Munts tomato paste	11.0
Munts tomato sauce	3.0

*ng/g of wet weight homogenized vegetable

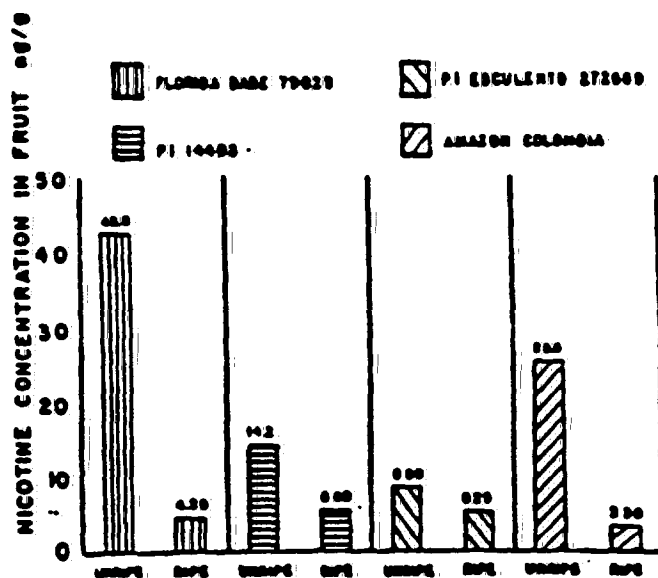


Figure 1
COMPARISON OF NICOTINE LEVELS
BETWEEN UNRIPE (green) AND RIPE (red) FRUIT
OF DIFFERENT TOMATO VARIETIES

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Acknowledgments

We would like to thank Leandro Ramos, University of Florida Agricultural Station, for his collaboration in this study. Without his help this research would have not been possible. This work was supported in part by a grant from the Council for Tobacco Research - USA, Inc. Special acknowledgment to Richard E. Bailey, M.D., for his interest and help throughout many years.

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CORRESPONDENCE

Letters to the Editor are considered for publication (subject to editing and abridgment), provided that they are submitted in duplicate, signed by all authors, typewritten in double spacing, and do not exceed 40 typewritten lines of manuscript text (including references). Submission of a letter constitutes permission for the Massachusetts Medical Society, its licensees, and its assignees to use it in the journal's various editions (print, data base, and optical disk) in anthologies, revisions, and any other form or medium. Letters should not duplicate similar material being submitted or published elsewhere, and they should not contain abbreviations. Financial associations or other possible conflicts of interest should always be disclosed.

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EXHAUSTIVE SMOKING

To the Editor: We found the mean urinary cotinine levels for smokers and for nonsmokers reported by Masuhara et al. (Sept. 27 issue)¹ to be surprisingly high. In our own study of 193 smokers (Table 1) we found a level of 1.11 µg per milligram of creatinine—similar to that obtained by other workers^{2,3} but almost eight times lower than the figure of 8.57 given by Masuhara et al. Similarly, the level of 0.68 that they reported for nonsmokers under normal living conditions is an order of magnitude higher than the figures obtained by other authors. In a model study of volunteers heavily exposed to tobacco smoke (25 ppm of carbon monoxide; 4.6 mg of total particulate matter per cubic meter for 80 minutes), Hoffmann and co-workers⁴ found a mean urinary cotinine level of 0.05 µg per milligram of creatinine. In a similar study (25 to 30 ppm of carbon

Table 1. Urinary Cotinine Excretion in Cigarette Smokers.*

No. of Cigarettes per Day	No. of Smokers	Urinary Cotinine Excretion
1-9	14	0.32±0.57
10-19	45	0.53±0.92
20-29	73	1.23±0.78
30-39	39	1.50±0.93
>40	22	1.75±1.10

*Measurements are based on 24-hour urine samples.

monoxide; 200 to 300 μg of nicotine per cubic meter for 180 minutes). Johnson and Leland (personal communication) confirmed these data.

This discrepancy is too large to be explained by differences between the Japanese and Westerners in nicotine intake or in urinary creatinine concentrations, and it suggests strongly that the radioimmunoassay used by the authors may not have been specific enough for cotinine, so that because of immunologic cross-reactions, substances related and not related to smoking were measured in addition to cotinine. The high background levels in the nonsmokers not exposed to tobacco smoke in the study of Matsukura et al. support this view. Before drawing far-reaching conclusions, the authors should compare their test system with those applied by other research groups working in this field.

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To the Editor: The data reported and the conclusions drawn by Matsukura et al. suggest to the reader that passive smoking may have "deleterious effects" on health. There are, however, procedural, statistical, and conceptual flaws in the study, which limit the way in which the data may be interpreted.

In the first place, no mention is made concerning the manner in which the subjects were selected. The authors do not state how subjects were solicited or whether any randomizing techniques were used to guard against systematic selection factors. Furthermore, no

mention is made concerning the matching of groups for such factors as medical history, socioeconomic status, occupation, and other variables related to a person's health. Indeed, no mention is made of whether persons with a prior smoking history were included in or removed from the nonsmoking group.

The authors note that within the group of smokers, there was a significant positive correlation between the number of cigarettes smoked and the urinary cotinine level. Since the correlation was 0.15, this indicates that the number of cigarettes smoked accounted for only 2.25 per cent of the variation in the levels of cotinine in those who smoked. In other words 97.75 per cent of the variation in the levels of cotinine was the result of error or other contributing factors that were not assessed.

Also unfortunate is the fact that a definitive control group was lacking in this study. It would have been informative if a group of nonsmoking subjects had been isolated from all forms of smoke for a long enough period to allow complete removal of nicotine and its metabolites. Analysis of this group's urinary cotinine levels would have demonstrated the radioimmunoassay technique's validity across extremes of the testing range, and whether passive smoking alone produced significantly higher cotinine levels than breathing totally smoke-free air for a known period.

Given the above criticisms, any conclusion drawn from this research is at best speculative.

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The above letters were referred to the authors of the article in question, who offer the following reply:

To the Editor: In response to Dr. Adlkofer et al., the antiserum we used was shown to be highly specific for cotinine.¹ We have measured urinary and plasma cotinine by our radioimmunoassay² and by gas-liquid chromatography³ and have found a high degree of correlation (Fig. 1). Concerning our urinary cotinine levels for smokers,³ we consider our results not surprisingly high but rather similar to those reported elsewhere.⁴⁻⁶ It is well known that cotinine excretion in smokers depends on such factors as the number of cigarettes smoked, the nicotine content of the brands, the manner of smoking, the metabolic efficiency of individual tobacco alkaloids, and the analytical procedures used. Urinary cotinine expressed per

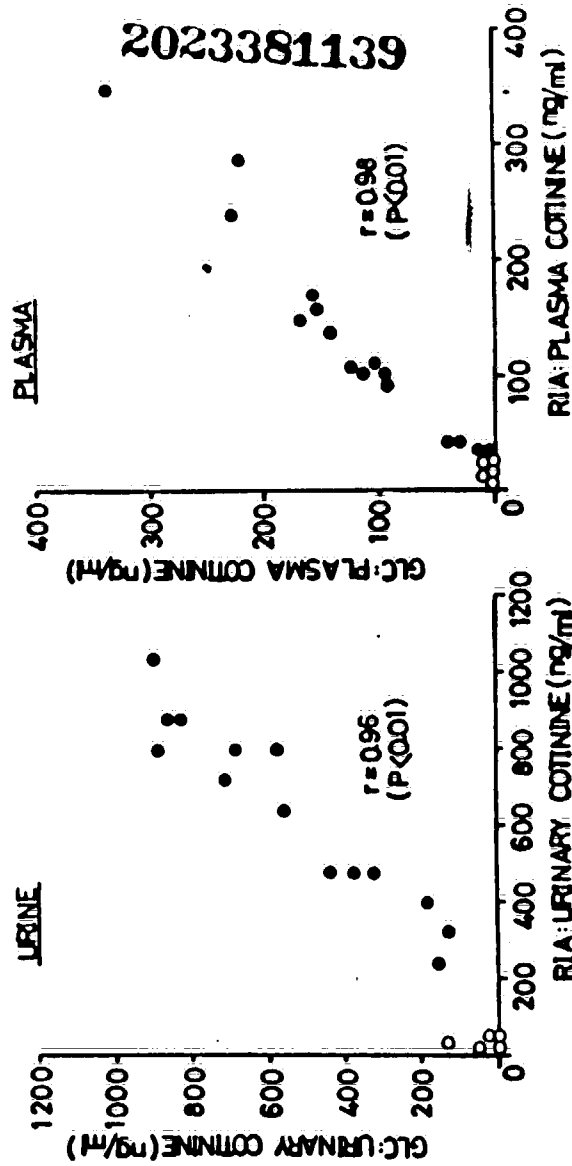


Figure 1. Urinary and Plasma Cotinine Levels in Smokers (●) and Nonsmokers (○). Measured by a Radioimmunoassay (RIA)¹ and by Gas-Liquid Chromatography (GLC).²

consider is also affected by body weight. We believe that if these errors are not taken into account, a simple comparison of different values seems impractical. Recently, we have also obtained rather low urinary cotinine values for smokers who smoke low-alkaline brands. Concerning our nonsmokers' urinary cotinine levels, the general prevalence of smoking, the fewer restrictions on smoking, and the small spaces of residence in Japan, resulting in close contact with smokers, may also contribute to the high levels.

In reply to Dr. Pillemer, we collected urine samples from residents living near the institutes where the study was performed. Mixing of groups was not done since we consider it unlikely that cotinine excretion is much influenced by the medical and socioeconomic backgrounds of nonsmokers who are exposed to the same degree of tobacco smoke. Moreover, it seems unlikely that a prior history of smoking among nonsmokers affects the results, unless we assume that cotinine prefer to go near smokers to inhale tobacco smoke. Concerning a significant but low correlation between the number of cigarettes and the urinary cotinine levels in smokers in our study, we believe that cotinine excretion depends on such multiplicative factors as mentioned above. Some heavy smokers are found to excrete less, to puff less, or to discard long butts, resulting in a low urinary cotinine level for the number of cigarettes smoked. A plausible effect of plasma bicarbonate and cotinine levels has been reported in smokers of more than 21 cigarettes per day,² although a high correlation has been observed in smokers of up to 20 cigarettes per day.³

We regret to have discovered an error in our article.¹ In Figure 1, the number of smokers who consumed 20 to 29 cigarettes per day should be 142, not 42.

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DIAGNOSIS OF PHEOCHROMOCYTOMA

To the Editor: The importance of the prevalence of the disease was not evaluated in the review of pheochromocytoma by Bravo and Gifford (Nov. 15 issue).¹ Although the prevalence of hypertension is high (15 to 20 per cent of the population),² the prevalence of pheochromocytoma in the Mayo Clinic series of hypertensive patients was only 0.04 per cent.³ Selection of hypertensive patients for a pheochromocytoma workshop is therefore critical to increase the likelihood of disease in the group of patients studied. If all hypertensive patients were screened with a test that was 96 per cent specific and 100 per cent sensitive,⁴ for every four true positive tests there

would be 200 false positive tests, and therefore, the predictive value of a positive test would be only 2 per cent. The economic impact of the testing would also be considerable.⁵ Unfortunately, the clinical syndromes that may be suggestive of pheochromocytoma (such as headache, sweating, and tachycardia) are common in other diseases. If, however, the prevalence of the disease in patients studied could be increased by clinical selection criteria to 1 per cent, for example, the predictive value of a positive test would increase to 35 per cent, since for every true positive test there would be two false positive tests.

Bravo and Gifford note that most cases of pheochromocytoma can be diagnosed by measurement of urinary catecholamine metabolites. The Mayo Clinic group recommended the urinary metanephrine test (96 per cent sensitive).⁶ In Bravo and Gifford's study,⁷ the urinary metanephrine test had a sensitivity (79 per cent) similar to that achieved by using plasma catecholamine levels above 2000 pg per milliliter, which Bravo and Gifford consider pathognomonic of pheochromocytoma. Higher sensitivity (96 per cent) for the urinary metanephrine test has also been claimed in another study.⁸ If plasma catecholamine levels above 950 pg per milliliter are considered abnormal, the sensitivity reaches 94 per cent in Bravo and Gifford's study. However, even in experienced hands, there is a risk of obtaining spuriously high plasma catecholamine levels in the range of 1000 to 2000 pg per milliliter in patients without pheochromocytoma, if blood specimens are not obtained with scrupulous care,⁹ thus impairing the specificity of the test. In the face of these falsely elevated results, patients without pheochromocytoma may be subjected to a subsequent invasive pharmacologic procedure, such as the clonidine suppression test, which as Bravo and Gifford concede, is not without side effects.¹⁰ Furthermore, use of the clonidine suppression test in this subset of patients with plasma catecholamine levels between 1000 to 2000 pg per milliliter has been incompletely studied.

We would like to stress that disease prevalence, as well as sensitivity and specificity, must be considered in the evaluation of screening tests.

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To the Editor: Bravo and Gifford found that the measurement of total plasma catecholamines either before or after the clonidine suppression test was a more sensitive and specific method of diagnosing pheochromocytoma than the measurement of urinary metanephrines. However, the diagnostic value of these three tests was evaluated with different subgroups of patients. How many patients underwent all three tests within 24 hours, and what were the sensitivity and specificity of the tests in these patients? We measured plasma catecholamines and urinary metanephrines within the same 24-hour period in 36 patients with surgically confirmed pheochromocytoma. The lowest value for urinary metanephrines was 0.916 mg per 24 hours, which is above the range of primary hypertension.

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NEVER SMOKER LUNG CANCER RISKS FROM EXPOSURE TO PARTICULATE TOBACCO SMOKE

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(Received 2 May 1987; Accepted 16 September 1987)

The average particulate environmental tobacco smoke (ETS) exposure of never and current smokers and the average lung cancer mortality rate for current smokers is estimated from empirical data. These estimates are used in a linear downward extrapolation of the lung cancer risk/mg of particulate ETS exposure for current smokers to calculate the average lung cancer risk for never smokers and the number of never smoker lung cancer deaths (LCD) in the U.S. in 1980 from exposure to particulate ETS. The estimated average daily inhaled particulate ETS exposure for never smokers is 0.62 mg/day for men and 0.28 mg/day for women. The average never smoker is estimated to retain 11% of the inhaled exposure, for a daily retained exposure of 0.07 mg for men and 0.03 mg for women. Other estimates are: a daily retained exposure for current smokers of 310 mg for men and 249 mg for women, a smoking-attributable lung cancer risk for current smokers in 1980 of 284 LCD/100,000 men and 121 LCD/100,000 women, and an annual retained-exposure lung cancer risk for never smokers of 0.64 LCD/100,000 men and 0.015 LCD/100,000 women. These risks and exposures estimate 12 lung cancer deaths among never smokers from exposure to particulate ETS: 8 among the 11.96 million male never smokers and 4 among the 28.85 million female never smokers in the U.S. in 1980. Conversely, between 655 and 3,610 never smoker lung cancer deaths are estimated from methods based on the average lung cancer risk observed in epidemiological studies of exposure to ETS. Three possible reasons for the discrepancy between the exposure and risk-based estimates are discussed: the excess risks observed in epidemiological studies are due to bias, the relationship between exposure and risk is supralinear, or sidestream tobacco smoke is substantially more carcinogenic than an equivalent exposure to mainstream smoke.

Introduction

Never smokers can be passively exposed to tobacco smoke at work, at home, and in public areas such as shopping centers and restaurants, particularly if the ventilation is poor. Concern over the average never smoker's lung cancer risk from exposure to "environmental" tobacco smoke (ETS) has grown considerably since a 1981 study reported an association between lung cancer in nonsmokers and marriage to a smoker (Hirayama, 1981). Since then, other epidemiological studies of the association between lung cancer among nonsmokers and ETS exposure from living with a smoker have been conducted in Japan (Akiba *et al.*, 1986), Greece (Trichopoulos *et al.*, 1983), Hong Kong (Chan & Fung, 1982; Koo *et al.*, 1985), Sweden (Pershagen *et al.*, 1987), Great Britain (Gillis *et al.*, 1984; Lee *et al.*, 1986), and the U.S. (Garfinkel *et al.*, 1981; Correa *et al.*, 1983; Buffler *et al.*, 1984; Kabat & Wynder, 1984; Garfinkel *et al.*, 1985; Dalager *et al.*, 1986; Brownson *et al.*, 1987; and Humble *et al.*, 1987). The

results of these studies have been used to estimate an average excess lung cancer risk for never smokers of 30% from ETS exposure (Blot & Fraumeni, 1986; NRC, 1986; Wald *et al.*, 1986; Wigle *et al.*, 1987). Alternatively, the risk can be estimated by downward extrapolation techniques based on the lung cancer risk for current smokers and the average exposure of current and never smokers. The former method is a risk-based estimate whereas the latter is an exposure-based estimate.

Linear Extrapolation

This study uses linear downward extrapolation to estimate the lung cancer risk and the number of U.S. lung cancer deaths for male and female never smokers in 1980 from exposure to ETS. The final estimate of the number of ETS-attributable never smoker lung cancer deaths requires four preliminary estimates:

1. The number of never smokers at risk,
2. The average tobacco smoke exposure of never smokers,

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3. The average tobacco smoke exposure of current smokers, and
4. The smoking-attributable lung cancer risk for current smokers.

The lung cancer risk for never smokers is estimated by dividing the lung cancer risk for active smokers by the ratio of the average tobacco smoke exposure of smokers and never smokers. The estimated lung cancer risk for never smokers is then used to predict the number of ETS-attributable never smoker lung cancer deaths in the U.S. in 1980. This is done by multiplying the estimated lung cancer risk for each sex by an estimate of the total U.S. population of never smokers of each sex ≥ 35 years of age. Appendix A lists the equations and parameters used in the linear extrapolation estimate.

Estimating the smoker/never smoker exposure ratio

The major difficulty with the extrapolation method is to determine the smoker/never smoker exposure ratio. Ideally, the exposure ratio is based on carcinogenically equivalent exposures, such that one unit of exposure for a never smoker has the same lung cancer risk as one unit of exposure for an active smoker. However, no carcinogenically equivalent measure of exposure exists because smokers and never smokers inhale different types of tobacco smoke. Smokers mostly inhale mainstream smoke produced at temperatures above 600°C and then drawn through the cigarette and filter, whereas the ETS inhaled by never smokers consists mostly of sidestream smoke formed between puffs at 350°C and, partly, of exhaled mainstream smoke. Due to the different combustion temperatures, the amount of specific carcinogens in mainstream and sidestream smoke differs. For example, measurements of two brands of modern filter cigarettes find that fresh sidestream smoke contains 42 times more N-nitrosodimethylamine and 1.5 times more benzo(a)pyrene, but 15% less catechol (a major cocarcinogen (Hecht *et al.*, 1981)) and 70% less N-nitrosonornicotine, by weight, than mainstream smoke (Adams *et al.*, 1985). The problem of the relative carcinogenicity of mainstream and sidestream smoke is exacerbated by the possibility of substantial differences in the composition of their gaseous and particulate phases; however, this has not been studied adequately. Overall, the relative carcinogenicity per unit weight of mainstream and sidestream smoke is not known, though there is some evidence to indicate that particulate sidestream smoke is more carcinogenic than mainstream smoke. An animal study cited by Wynder and Hoffman (1967) finds more skin cancers among shaved mice painted with particulate sidestream versus mainstream condensates. A series of four Ames mutagenicity assays finds particulate sidestream smoke to be over ten times more mutagenic than an equivalent amount, by weight, of mainstream smoke in

one test series, though there is little difference in the other three tests (Lofroth & Lazaridis, 1986).

In the absence of a measure of the carcinogenicity of mainstream and sidestream smoke, this study uses the current and never smoker's retained exposure to particulate ETS to estimate the smoker/never smoker exposure ratio. The exposure estimate adjusts for the dilution of sidestream smoke by ambient air. As a first approximation, the carcinogenicity of mainstream and sidestream particles is assumed to be equal (the effect of assuming a greater carcinogenicity for sidestream smoke is discussed later). Exposure to the gas phase of mainstream and sidestream smoke is not included because exposure to the gas phase, without concurrent exposure to tobacco smoke particulates, has not been found to cause lung cancer (Hoffmann *et al.*, 1978; SG, 1982). However, it is possible that future research may establish a significant carcinogenic role for the gas phase.

The smoker/never smoker exposure ratio is also based on the retained exposure (the amount of particulate by weight deposited in the lungs) instead of the inhaled exposure. A significant proportion of the latter is immediately exhaled and, consequently, has no effect on carcinogenesis. Hiller *et al.* (1982) experimentally determine in 11% particulate retention rate for never smokers exposed to sidestream smoke. Similar results have been found for other particulates in the size range of sidestream smoke (Davies *et al.*, 1972; Heyder, 1982). Conversely, the average active smoker retains between 47% and 96% of inhaled mainstream smoke, with most estimates falling above 70% (Dalhamm, 1968; Hoegg, 1972; Corn, 1974; First, 1984). The higher deposition rate for active smokers is thought to result either from deeper inhalation (Muir, 1974), hygroscopic growth and coagulation (Hiller *et al.*, 1982), or from electrical charges in mainstream smoke (Stober, 1984).

ETS exposure and cotinine. Tobacco smoke exposure can also be determined from blood, urine or saliva levels of cotinine — a nicotine metabolite. Cotinine appears, at first, to be a more accurate measure of exposure than retained particles. The latter can only be indirectly estimated for never smokers from exposure to all respirable particles, which include dust, pollen, and other aerosols. In contrast, cotinine is an accurate and specific indicator of tobacco smoke exposure because it is the only important source of exposure to nicotine. Cotinine also measures dose (the amount of a tobacco constituent metabolized by the body), whereas an estimate of retained particles only measures exposure. Unfortunately, though several studies show that cotinine levels in body fluids can accurately differentiate between never smokers with high, moderate and low ETS exposure (Jarvis *et al.*, 1984; 1985) or between nonsmokers and current smokers (Wald *et al.*, 1984), cotinine levels in never and current smokers are not directly comparable. This is

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Table 1. Estimated number of never, ex-, and current smokers \geq age 35 in 1980 in the U.S.*

Smoking Status	Men		Women		Total
	Number	Percent	Number	Percent	
Never	11,960,000	27.03	28,846,000	55.66	40,806,000
Ex-	15,314,000	34.62	7,774,000	15.00	23,088,000
Current	16,965,000	38.35	15,201,000	29.34	32,166,000
Total	44,239,000	100.00	51,821,000	100.00	

*Based on U.S. Census figures for the total male and female population \geq 35 years of age (USDC, 1982) and the percentage of never smokers, ex-smokers and current smokers of all races \geq 35 from the 1979/1980 National Health Interview Survey.

partly because the half-life of cotinine is substantially shorter for current than never smokers (Kyrematen *et al.*, 1982; Lynch, 1984; Sepkovic *et al.*, 1986), so that cotinine levels could underestimate the relative exposure received by the lungs of smokers. However, the major problem with using cotinine to determine relative exposures is due to the occurrence of nicotine in a protonated form in the particulate phase of the mainstream smoke inhaled by smokers but in an unprotonated form in the gas phase of the sidestream smoke inhaled by nonsmokers (Eudy *et al.*, 1986; IARC, 1985). Consequently, cotinine or nicotine levels in smokers measure the lung's particulate exposure, whereas these levels in nonsmokers largely measure nasal and pharyngeal exposure to gas phase constituents with a similar retention rate to that of nicotine. The two estimates of exposure are not comparable because they differ both by site and type of exposure. Nonsmokers should also absorb a higher percentage of inhaled nicotine than smokers because the unprotonated nicotine of sidestream smoke is absorbed more rapidly than the protonated nicotine of mainstream smoke (IARC, 1985).

Data Sources and Assumptions

Though superficially simple, the calculation of each of the four preliminary estimates is based on a large number of estimated parameters. These parameters are obtained from published data and analyses of the 1970 (NCHS, 1970) and 1979/1980 National Health Interview Surveys (NCHS, 1981). Due to the large sample size of the National Health Interview Surveys (the 1970 and 1979/1980 NHIS contain smoking data for a sample of 74,451 and 37,604 individuals \geq 17 years of age, respectively), these surveys provide the best available estimates of the number of never, ex-, and current smokers by occupation, age, and sex (Table 1). Studies of ambient particulate ETS levels in white-collar workplaces in the U.S. are identified from the Building Performance Database, an on-line database accessible through national data networks (Sterling *et al.*, 1985). Most of the identified studies were conducted by the National Institute for Occupational Safety and Health.

All four preliminary estimates are based on estimated average exposures and risks for never and current smokers in 1980. The best exposure-based risk estimate would compare cumulative lifetime exposure for never and ever smokers, because the lifetime exposure of many ex-smokers exceeds that of current smokers. Unfortunately, no cumulative lifetime exposure data for a representative sample of ever and never smokers exists. The estimated risk and the number of excess lung cancer deaths are given for never smokers age 35 and over because almost all lung cancer deaths occur in this age group. However, the average exposure is calculated for never smokers \geq 17 years of age. ETS exposure is more frequent among young adults (Friedman *et al.*, 1983), and this early exposure could latently affect the lung cancer risk.

In addition to many minor assumptions concerning the accuracy and representativeness of the data, the final estimate of the number of never smoker lung cancer deaths from exposure to particulate ETS is based on four major assumptions:

1. The carcinogenicity of tobacco smoke depends upon exposure to the particulate phase,
2. The lung cancer risk per unit exposure to mainstream and sidestream particulate tobacco smoke is the same,
3. The relationship between risk and each unit of exposure is approximately linear, and
4. There is no low exposure threshold where the lung cancer risk falls to zero.

Calculation of the Linear Extrapolation Estimate

The following three sections calculate the average particulate ETS exposure for never smokers and current smokers as well as the lung cancer risk for current smokers. The population of never smokers, estimated from the 1979/1980 National Health Interview Survey, is given in Table 1.

Never Smoker's Average Particulate ETS Exposure

Almost all particulate ETS exposure occurs indoors

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Table 2. Estimated time budgets (hours/day)^a in 1980 in the U.S.

Location	Employed			All Women	All Men
	Women	Men	Housewives		
In homes (their own and others)	16.3	14.1	21.3	18.6	15.7
Workplace	4.7	6.1	0.0	—	—
Places of business and other locations	1.3	1.4	1.5	1.5	1.3
Restaurants and bars	0.3	0.5	0.1	0.2	0.4
Outside of homes and in transit	1.4	1.9	1.1	— ^b	—
	24.0	24.0	24.0		

^aThe 1965 data from Szalai (1972) are adjusted to account for a 9% drop in the average hours worked between 1965 and 1980 (USDC, 1981). The reduction in work time, 0.6 hours for men and 0.5 hours for women, is divided among the time spent in the four other locations.

^bNo exposure is assumed for this category.

because of low indoor ventilation rates and because adults spend over 85% of their time indoors (Szalai, 1972). The average never smoker's exposure to particulate ETS depends on 1) the average inhalation rate and, for each indoor location, 2) the time spent there, 3) the average particulate ETS level, and 4) the proportion of never smokers exposed.

1. Inhalation rate

Data from Altman and Ditmer (1971) indicate an average inhalation volume of 1.08 m³/hour for men and 0.62 m³/hour for women, based on the average volume of air inhaled at rest and during light work.

2. Time spent in five locations

The results of a 1965 44-city time budget study (Szalai, 1972) are used to estimate the average daily time spent by never smokers in five locations: home, work (including time before and after work and during lunch), places of business and other locations, restaurants and bars, and outside the home and in transit. The time spent in restaurants and bars is determined separately, because they sometimes have very high ambient particulate ETS levels. Unfortunately, the original data do not permit dividing time into indoor and outdoor hours — time spent in both "places of business and other locations" and "outside of home and in transit" includes indoor and outdoor locations. To simplify matters, we assume that all time spent in "places of business and other locations" is indoor time where particulate ETS exposure occurs and all time spent "outside of home and in transit" is outdoor time with no particulate ETS exposure.

Table 2 estimates the average time spent in each location by employed men, employed women, and housewives. The time budget for housewives is used to estimate the time budget for all nonemployed men and women ("nonemployed" includes individuals actively seeking work, homemakers, and retirees). Time spent in nonwork locations is adjusted for the proportion of nonemployed adults because, according to time budget results, housewives spend more time in places of busi-

ness and less time in restaurants and bars than do employed individuals. The 1979/1980 National Health Interview Survey estimates that 19.1% of male and 49.2% of female never smokers are nonemployed. Table 2 also gives the employment-weighted time spent in each nonwork location.

3. Indoor particulate ETS levels

Indoor particulate ETS exposure is indirectly estimated from on-site measurements of total or respirable suspended particles. This method requires an adjustment for background (nonsmoking-related) particulate levels. Background measurements should be taken indoors when no one has smoked for several hours but when all other conditions are the same as during periods of smoking (these criteria are rarely met). If there are no indoor background measurements, outdoor measurements are used as a crude estimate of the indoor particulate level in the absence of smoking.

Estimated particulate ETS levels in restaurants and bars. The average particulate level (unadjusted for background levels) in 27 restaurants, bars and entertainment facilities is 0.30 mg/m³ (see Table 3). The average indoor particulate ETS level, after adjusting for the average background particulate level of 0.04 mg/m³, is 0.26 mg/m³.

Estimated particulate ETS levels in places of business. The estimated average particulate ETS level in offices (see below) is also used for the time budget category "places of business and other locations" (banks, shopping centers, etc.).

Estimated particulate ETS levels in the workplace. Workplace particulate levels are available for restaurants, bars, offices, and service buildings. It is impossible to estimate particulate ETS exposures from particulate levels in indoor blue-collar workplaces because of high background particulate levels from industrial activities. The average particulate ETS exposure should be less for blue-collar than white-collar never smokers because blue-collar workplaces, compared to offices, are better ventilated in order to re-

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Table 3. Particulate levels (mg/m^3) in bars, restaurants and entertainment facilities in the U.S.

Reference	Premise	Measurement Type	#	Mean	Background (# measurements)
Cuddeback <i>et al.</i> , 1976	2 Taverns	TPM ^a	5	0.445	(none)
Elliot & Rowe, 1975	3 Arenas	TPM	19	0.367	0.07 (2 indoors)
First, 1984	3 Taverns	nd ^b	nd	0.543	(none)
	3 Restaurants	nd	nd	0.220	(none)
Repace & Lowrey, 1980	17 Entertainment facilities ^c	RSP ^d	20	0.243	0.04 (4 indoors)
Average: ^e				0.30	
Adjusted for background levels: ^f				0.26	

^aTotal particulate matter.^bNo data given.^cThree bars, seven restaurants, and one each of a lodge, bar and grill, firehouse bingo game, church bingo game, inn, bowling alley, and an arena. Active smoking occurred during the time of all measurements.^dRespirable suspended particles.^eThe average is determined by weighting for the number of premises in each study.^fAdjusted for Repace and Lowrey's (1980) background level of $0.04 \text{ mg}/\text{m}^3$. The background level of Elliot and Rowe is not used because it is for arenas which make up only a small proportion of high exposure workplaces.

duce dust and fume exposure and because smoking is prohibited in a higher percentage of blue-collar workplaces (NICSII, 1978). However, we assume that exposed blue-collar workers receive the same particulate ETS exposure as white-collar office workers.

Table 4 summarizes the results of twenty studies of particulate levels in office or service buildings in the U.S. The mean total and respirable particulate levels are $0.08 \text{ mg}/\text{m}^3$ and $0.068 \text{ mg}/\text{m}^3$, respectively. The overall mean particulate level, ignoring the difference between total and respirable particulates, is $0.076 \text{ mg}/\text{m}^3$. The mean level, limited to twelve studies where active smoking was reported to occur during the time of measurement, is also $0.076 \text{ mg}/\text{m}^3$.

Outdoor background levels, reported by seven studies, average $0.053 \text{ mg}/\text{m}^3$ and range from 0.01 to $0.1 \text{ mg}/\text{m}^3$. The indoor background level in the absence of active smoking is $0.035 \text{ mg}/\text{m}^3$ in two studies of offices (Parker *et al.*, 1983; Collett, 1985) and $0.037 \text{ mg}/\text{m}^3$ in one study of an office and two libraries (Repace and Lowrey, 1980).

The minimum background outdoor particulate level of $0.01 \text{ mg}/\text{m}^3$ is used to conservatively estimate the indoor particulate ETS level because indoor particulate levels for 13 studies are below the outdoor average of $0.053 \text{ mg}/\text{m}^3$ while in eight studies the indoor particulate level is below the indoor background level of approximately $0.035 \text{ mg}/\text{m}^3$. Therefore, up to $0.066 \text{ mg}/\text{m}^3$ ($0.076 - 0.01$) of airborne particles in office and service buildings could be from tobacco smoke, assuming no other indoor sources of particles.

Estimated particulate ETS levels in residences. Five field studies measure the effect of at least one smoker on the 24-hour particulate level in residences. Table 5 shows that one smoker increases the hourly particulate level over background levels (homes with no smokers)

by an average of $0.015 \text{ mg}/\text{m}^3$, while two smokers increase the particulate level by an average of $0.042 \text{ mg}/\text{m}^3$. The latter average is assumed to represent all residences with two or more smokers.

The average home exposure is weighted by the proportion of never smoking respondents to the 1970 National Health Interview Survey who reported living with one versus two or more smokers. The weighted average hourly particulate ETS level in the residences of never smokers is approximately $0.02 \text{ mg}/\text{m}^3$ for both sexes.

4. Proportion of never smokers exposed in each location

All never smokers are assumed to be exposed in "restaurants and bars" and "places of business and other locations." The proportion exposed at work is estimated from the occupational distribution of never smokers while the proportion exposed at home is estimated from the proportion of never smokers who report living with a current smoker (spouse, relative, friend, etc.).

Proportion of never smokers exposed at work. Table 6 gives the 1979/1980 National Health Interview Survey employment distribution of never smokers age 17 and older. Occupations are grouped by the likelihood of particulate ETS exposure. Students are categorized as working in indoor white-collar environments, while blue-collar workers are separated into outdoor and indoor workers. All white-collar, indoor blue-collar, and restaurant and bar employees are assumed to be exposed to particulate ETS at work, while nonemployed individuals and outdoor workers are assumed to receive no workplace exposure. In total, 66.7% of all male and 49.3% of all female never smokers are estimated to be exposed to particulate ETS at work.

Proportion of never smokers exposed at home. The

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Table 4. Particulate levels (mg/m³) in white-collar workplaces in the U.S.

Reference	Use	Building		Measurement			Date	Mean
		Smoking ^a	Employees ^b	Type	#	Length		
Blake <i>et al.</i> , 1981	Store	nd	nd	TPM	10	nd	Nov/79	0.019
Chrostek, 1979	Office	nd	30	RSP	1	7 hr	May/79	0.030
Chrostek & Moshell, 1982	Office	nd	100	TPM	5	8 hr	Aug/81	0.120
Collett, 1985	Office	Y	6 ^c	RSP	8	40 min	Jul/85	0.050
Cornwell <i>et al.</i> , 1981	Office	Y	>100	RSP	28	2 min	Nov/80	0.048
Gorman, 1980	Office	nd	6	TPM	nd	nd	Jul/80	0.11 ^d
Gunter & Thoburn, 1985	Office	nd ^e	nd	TPM ^f	7	6.5 hr	Nov/84	0.164
Hicks, 1981a	Office	Y	nd	nd	3	nd	Mar/80	0.047
Hicks, 1981b	Office	nd	40	TPM	2	6 hr	Dec/80	0.055
Hodgson & Morley, 1983	Office	nd	41	RSP	3	nd	Mar/83	0.025
Hollett, 1979	Office	nd	nd	TPM	21	nd	Jul/79	0.143 ^h
Moschandreas <i>et al.</i> , 1980	Office	Y	150	TPM	3	24 hr	nd	0.030 ⁱ
	Office	Y	100	TPM	3	24 hr	nd	0.038 ^j
Neal <i>et al.</i> , 1978	Hospital	Y	nd	TPM	41	48 hr	Aug-Feb	0.030
Parker <i>et al.</i> , 1983	Office	Y	100	TPM	2	8 hr	Feb/83	0.032
	Office	Y	16	TPM	3	8 hr	Feb/83	0.094
Repace & Lowrey, 1980	Hospital waiting room	Y	nd	RSP	1	12 min	Mar	0.187
Salisbury, 1979	Stock Exchange	Y	nd	TPM	3	5 hr	Oct/78	0.287
Salisbury <i>et al.</i> , 1982	Office	Y	500	TPM	8	6 hr	Mar/81	0.038
Tharr, 1980	Office	nd	100	TPM	2	7 hr	Jun/80	0.060
Thompson <i>et al.</i> , 1973	2 stores	nd	nd	TPM	nd	nd	Nov/71	0.083
Turiel <i>et al.</i> , 1981	Office	Y	nd	TPM	nd	12 hr	nd	0.031 ^k
Average:								0.076
Adjusted for Background Level (see text):								0.066

^aActive smoking while particulate levels were sampled.^bOn the floor(s) where measurements taken.^cRespirable suspended particles.^dIn immediate area of sampler, two of the six staff were smokers.^eClose to office copier.^fNo data given.^gTotal particulate matter.^hMajor construction site across the street.ⁱBased on 24 hour sample. Maximum recorded 0.057 mg/m³.^jBased on 24 hour sample. Maximum recorded 0.130 mg/m³.Table 5. Effect of smoking on 24-hour respirable suspended particle (RSP) levels (mg/m³) in residences in the U.S.

Reference	Mean RSP Level in Homes with			Increase due to	
	No Smokers	1 Smoker	2 Smokers	1 Smoker	2 Smokers
Spengler <i>et al.</i> , 1981	0.024	0.037	0.052	0.012	0.027
EPRI, 1984	0.024	0.043	0.075	0.019	0.051
Hosein & Corey, 1986 ^a	0.038	0.053	0.080	0.015	0.042
Lebowitz <i>et al.</i> , 1984	0.018	0.033 ^b	—	0.015	—
Spengler <i>et al.</i> , 1985	0.028	—	0.074 ^b	—	0.046
Average:				0.015	0.042

^aAverage of reported winter and summer means.^bNumber of resident smokers not given in reference. The results have been assigned to the most probable category, on the basis of the results of the other studies.

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Table 6. 1979/1980 National Health Interview Survey estimate of the occupational distribution of never smokers \geq age 17 in the U.S.

Occupation	Men (%)	Women (%)
Indoor white-collar ^a	43.3	41.0
Indoor blue-collar ^b	21.6	6.2
Restaurants and bars ^c	1.8	2.1
Outdoor workers ^d	14.2	1.5
Not employed ^e	19.1	49.2
	100.0	100.0

^aIncludes students and professional, managerial, technical, clerical and service occupations.

^bIncludes industrial and warehousing occupations.

^cIncludes waiters and waitresses, entertainers, bartenders, busboys, recreation and amusement attendants, pub and food service workers.

^dIncludes construction, agriculture, forestry and fishing occupations.

^eIncludes retired, homemakers and unemployed.

1970 National Health Interview Survey estimates that 29.6% of male and 35.7% of female never smokers age 17 and over live with a current smoker. Since the 1979/1980 National Health Interview Survey does not include smoking information for all household members, it cannot be used for this purpose. However, using data for 1970 should overestimate the proportion of never smokers exposed at home in 1980 because the number of active smokers has declined 6% for men and

2% for women between 1970 and 1980 (Weinkam & Sterling, 1987).

Estimated particulate ETS exposure

Table 7 summarizes the average never smoker's inhalation exposure to particulate ETS. The average daily inhaled particulate ETS exposure is 0.62 mg for male and 0.28 mg for female never smokers. The daily retained particulate ETS exposure, based on an 11% retention rate, is 0.07 mg for male and 0.03 mg for female never smokers.

Current Smoker's Average Exposure

The average smoker's daily exposure to particulate tobacco smoke is assumed to equal the average number of cigarettes per day consumed multiplied by the average tar delivery per cigarette. For simplicity, the current smoker's particulate ETS exposure is not included because it is only a small fraction of the current smoker's total particulate tobacco smoke exposure. An average consumption of 29.3 cigarettes per day for current smokers is calculated by dividing the 1979/1980 average of 626.5 billion cigarettes sold in the U.S. (Maxwell, 1981) by the 1979/1980 National Health Interview Survey estimate of 58.5 million current smokers age 17 and over. The average of 29.3 cigarettes per day is used for both sexes because there is little difference in the 1979/1980 National Health

Table 7. Estimate of the average never smoker's inhalation exposure of particulate environmental tobacco smoke (ETS) (mg/day) in 1980 in the U.S.

Location	Respiration Rate/hour	Ambient ETS mg/m ³	Hours of Exposure	Proportion Exposed	Weighted Exposure
Men					
Home	1.08	0.02	15.7	0.296	0.100
Rest/Bar ^a	1.08	0.26	0.4	1.000	0.112
Other ^b	1.08	0.066	1.3	1.000	0.093
Work					
White-collar	1.08	0.066	6.1	0.433	0.188
Blue-collar	1.08	0.066	6.1	0.216	0.094
Rest/Bar	1.08	0.26	6.1	0.018	0.031
No Workplace Exposure ^c	—	—	—	0.333	0.000
					Total 0.62
Women					
Home	0.62	0.02	18.6	0.357	0.082
Rest/Bar	0.62	0.26	0.2	1.000	0.032
Other	0.62	0.066	1.5	1.000	0.061
Work					
White-collar	0.62	0.066	4.7	0.410	0.079
Blue-collar	0.62	0.066	4.7	0.062	0.012
Rest/Bar	0.62	0.27	4.7	0.021	0.016
No Workplace Exposure	—	—	—	0.501	0.000
					Total 0.28

^aRestaurants and bars.

^bPlaces of business and other locations.

^cUnemployed, retired, homemakers, and outdoor workers.

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Table 8. National Mortality Followback Survey estimate of the number of lung cancer deaths (LCD) among never smokers in the U.S. in 1980.

Age	LCD/100,000 ^a	Never Smokers (100,000) ^b	Estimated LCD
Men			
35-44	2.3	37.08	85.3
45-54	3.5	25.68	89.9
55-64	33.4	22.91	765.2
65-74	63.8	19.80	1,263.2
75-84	87.9	11.05	971.3
85+	100.0 ^c	3.04	304.0
			Total: 3,479
Women			
35-44	0.4	62.60	25.0
45-54	2.8	61.30	171.6
55-64	10.9	61.30	668.2
65-74	18.7	55.96	1,046.5
75-84	48.4	36.87	1,784.5
85+	60.0 ^c	10.27	616.2
			Total: 4,312

^aFrom Enstrom & Godley (1980).

^bEstimated from the 1979/1980 National Health Interview Survey age distribution of never smokers of all races and the U.S. Census (USDC, 1982) estimate of the population in 1980.

^cEstimated from the American Cancer Society study (Garfinkel, 1981) as the rates for this age group were not given by the National Mortality Followback Survey (Enstrom & Godley, 1980). The American Cancer Society never smoker lung cancer rates are 94.8 for men and 52.4 for women 85-89 years of age. These rates are increased slightly to account for higher rates among people older than 89 and for the higher incidence of occupational exposures for the entire U.S. population.

Interview Survey estimate of the average consumption by sex; the average female smoker consumes 96% of the average for male smokers.

The 1980 sales-weighted average tar delivery per cigarette, excluding particulate nicotine, is 13.2 mg/day (Tobacco Institute, 1981). This average is used in the risk estimates for men. However, data from the American Cancer Society study show that women inhale approximately 20% less than men (Hammond, 1966). We assume that women in 1980 still inhale 20% less than men, and are thus exposed to 10.6 mg/day. The average daily particulate tobacco smoke exposure is, consequently, 387 mg for male and 311 mg for female smokers. The daily retained exposure, based on an 80% retention rate, is 310 mg for male and 249 mg for female smokers, excluding exposure to ETS.

Current Smoker's Average Lung Cancer Rate

In 1980 there were 75,362 male and 28,210 female lung cancer deaths among individuals age 35 and over (NCHS, 1986). The number of lung cancer deaths among current smokers cannot be directly determined because the smoking status of decedents is not noted on death certificates. The number of current smoker lung cancer deaths from tobacco smoke exposure is

obtained by subtracting, from the total number of lung cancer deaths, an estimate of the number of lung cancer deaths among never smokers, ex-smokers, and current smokers due to nonsmoking causes.

Lung cancer deaths among never smokers

Age-specific lung cancer death rates are available for white never smokers from the National Mortality Followback Survey (Enstrom & Godley, 1980). The use of these rates assumes identical rates for non-whites and no change in the lung cancer death rate for never smokers between 1966-1968 and 1980. These lung cancer death rates, multiplied by the age-specific number of never smokers in 1980, estimate 3,479 male and 4,312 female never smoker lung cancer deaths in the U.S. in 1980 (Table 8).

Lung cancer deaths among ex-smokers

The number of lung cancer deaths among the 23.09 million ex-smokers in 1980 is estimated from the average lung cancer relative risk for current versus ex-smokers in two cohort studies: the American Cancer Society study (Hammond, 1964) and the U.S. Veterans study (Rogot & Murray, 1980). The male risk ratios are also used for women because none of the cohort studies reports the risk for all ex- and current female smokers.

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The average lung cancer risk for all current versus all ex-smokers is 2.26, based on a risk of 1.67 in the American Cancer Society study and 2.85 in the Veterans study. The average ratio of 2.26, combined with the 1979/1980 National Health Interview Survey estimates of the number of current and ex-smokers, predicts 20,517 male and 4,410 female ex-smoker lung cancer deaths.

The difference in the risks for ex-smokers compared to current smokers in the two studies is probably due to the higher percentage of long-term ex-smokers, with lower lung cancer rates than short-term ex-smokers, in the Veterans study. All ex-smokers in the Veterans study had not smoked for a minimum of twelve years compared to a minimum of 34 months for ex-smokers in the American Cancer Society study. The American Cancer Society results may be more appropriate here because the National Health Interview Survey estimate of the number of ex-smokers includes both short and long-term ex-smokers.

Current smoker lung cancer deaths not attributable to smoking

The number of nonsmoking-attributable current smoker lung cancer deaths is estimated by assuming that smokers would experience the mortality rates of the National Mortality Followback Survey never smokers if they did not smoke. These mortality rates, applied to the 1979/1980 National Health Interview Survey estimate of the sex and age-specific population of current smokers, estimate 3,111 male and 1,094 female lung cancer deaths.

Smoking attributable lung cancer risk for current smokers

The number of smoking-attributable lung cancer deaths among male current smokers over 35 is 48,255, obtained from subtracting 3,479 never smoker, 20,517 ex-smoker, and 3,111 nonsmoking-attributable lung cancer deaths among current smokers from the 1980 total of 75,362 male lung cancer deaths. The same method estimates 18,394 smoking-attributable lung cancer deaths among female current smokers over 35 in 1980. Given 16.965 million male and 15.201 million female current smokers over 35 in 1980, this estimates a smoking-attributable lung cancer risk of 284 lung cancer deaths (LCD)/100,000 male and 121 LCD/100,000 female current smokers over age 35. Repace and Lowrey (1985) estimate a rate of 316 LCD/100,000 male or female smokers for use in their linear extrapolation estimate. However, they assume that all smoking-attributable lung cancer deaths occur among current smokers only (no deaths among ex-smokers), they do not adjust for occupational or other causes of lung cancer among smokers, and they include cancers of the larynx and other non-lung sites.

Lung Cancer Risk for Never Smokers From Particulate ETS Exposure

The estimated daily retained exposure is 310 mg for male and 249 mg for female current smokers and 0.07 mg for male and 0.03 mg for female never smokers. The male smoker's retained exposure is 4,429 times greater than the average male never smoker's retained exposure. Given a smoking-attributable lung cancer death rate for male current smokers of 284 LCD/100,000, the average male never smoker's lung cancer risk is 0.064 LCD/100,000, which predicts approximately 8 lung cancer deaths from exposure to particulate ETS among the 11.96 million male never smokers in 1980. The same method estimates a risk of 0.015 LCD/100,000 female never smokers, which predicts 4 lung cancer deaths from exposure to particulate ETS among the 28.85 million female never smokers in 1980.

Reliability of the Estimate

The linear extrapolation estimate of 12 never smoker lung cancer deaths in 1980 from exposure to particulate ETS is based on a large number of unverifiable assumptions and parameter estimates. Due to the large number of assumptions, it is neither meaningful nor possible to calculate upper and lower confidence limits. However, the reliability of each of the four preliminary estimates is assessed below. Evidence indicates that several of the preliminary estimates are more likely to result in an overestimate rather than an underestimate of the true number of never smoker lung cancer deaths. However, a plausible upper estimate is calculated for three of the four preliminary estimates. These upper estimates are used to calculate a maximum final estimate, given the major assumptions of the linear extrapolation method.

Number of Never Smokers

The final estimate of the number of never smoker lung cancer deaths increases if the estimated number of never smokers increases. The present estimate of 11.96 million male and 28.85 million female never smokers is based on self-reported smoking status, and is probably an overestimate of the number of never smokers. Two-stage interview studies show that approximately 5% of self-reported never smokers are actually ex- or current smokers (NRC, 1986). For this reason, the estimated number of never smokers is not increased when calculating the upper estimate.

Never Smoker's Average Exposure

The estimated number of never smoker lung cancer deaths increases if the average never smoker's exposure increases. Several factors suggest that our estimated exposure *overestimates* the true exposure. For

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example, many of the measurements of total or respirable particles are for workplaces which probably had higher than average particulate ETS levels. Most buildings in the Building Performance Database were studied in response to occupant complaints about "building illness" or poor indoor air quality. Even though tobacco smoke may not be an important factor in building illness (Sterling *et al.*, 1987), it could affect the occupants' perception of the air quality and lead to more complaints in buildings with high versus low particulate ETS levels. In addition, all indoor blue- and white-collar workers are assumed to be exposed to particulate ETS at work, even though smoking is prohibited in 11% of white- and 28% of blue-collar workplaces (NICS, 1978). Interview studies also show lower workplace exposure rates than the estimated rate of 66.7% for male and 49.3% for female never smokers. For example, only 29.4% of the control group of female never smokers in a case-control study report workplace exposure during the past 25 years (Garfinkel *et al.*, 1985). In a 1979/1980 questionnaire survey of 37,881 never smokers, only 40.4% of both sexes combined reported ETS exposure in "small spaces" such as at work (Friedman *et al.*, 1983).

Two field studies contain enough information to calculate the average inhaled particulate ETS exposure for men. Both of these studies estimate average particulate ETS exposures that are within six percent of the comparable estimates given in Table 7. The studies use personal monitors, carried by subjects for several days, to determine average exposures to respirable suspended particles. The difference between the average personal and background (outdoor) exposures to respirable suspended particles in a study of 48 subjects, none of whom live with a smoker, is 0.019 mg/m³ (Sexton *et al.*, 1984). If particulate ETS accounts for all of the difference in respirable suspended particles, then the average 24-hour particulate ETS exposure at a male respiration rate of 1.08 m³/hour, is 0.49 mg/day. For comparison, our estimate of the average daily inhaled exposure of male never smokers with no home exposure is 0.52 mg/day (see Table 7). Spengler *et al.* (1985) determine personal exposures to respirable suspended particles for 101 volunteers in two industrial towns in Tennessee. The difference between the average personal exposure and the background (outdoor) level is 0.024 mg/m³. This predicts an average daily particulate ETS exposure of 0.62 mg/day, which is identical to our estimate for men.

Repace and Lowrey (1985) estimate an average inhaled exposure for either sex of 1.43 mg/day, based on modeling indoor smoking, occupancy, and ventilation rates, which is 3.18 times the average of our estimates for men and women. There are several possible causes for the difference. For example, Repace and Lowrey assume that all never smokers are actively employed and, therefore, possibly exposed at

work. In contrast, the 1979/1980 National Health Interview Survey estimates that approximately 40% of all never smokers are not employed. Furthermore, Repace and Lowrey calculate the average ambient ETS level in the workplace from the average of modeled levels in low-exposure and high-exposure workplaces. Their model estimates an ambient particulate level (from tobacco smoke alone) of 0.17 mg/m³ for low-exposure workplaces such as offices and of 0.42 mg/m³ for high-exposure workplaces. The particulate level for the latter is representative of measured levels in very smoky workplaces such as taverns, bars, and dance halls. The average for the two types of workplaces is 0.30 mg/m³, which is higher than all of the measured levels for white-collar workplaces (see Table 4) and higher than the average for restaurants, bars and other entertainment facilities (see Table 5). It may not be appropriate to average the estimate for low and high-exposure workplaces, because the 1979/1980 National Health Interview Survey estimates that approximately twenty times as many never smokers work in low-exposure workplaces such as offices than in high-exposure workplaces such as bars and taverns.

There is no plausible average upper estimate for the inhaled exposure which is substantially greater than the estimates given in Table 7, which is also based on field measurements of ambient particulate levels. However, an arbitrary upper estimate can be made by doubling the previous estimates. Thus, the upper inhaled estimates are 1.24 mg/day for male and 0.56 mg/day for female never smokers. These give average retained exposures of 0.14 mg/day for male and 0.06 mg/day for female never smokers.

Current Smoker's Average Exposure

The estimated number of never smoker lung cancer deaths increases if the average smoker's true exposure, derived from the average tar delivery per cigarette multiplied by the average cigarette consumption, is less than estimated.

The average tar delivery per cigarette is based on machine smoked deliveries. The machine smoking standard of one two-second 35 ml puff/minute was established over 30 years ago to reflect the smoking habits of that time. Since then, several studies indicate that smokers partially compensate for a decline in the nicotine delivery per cigarette by smoking each cigarette more intensely (Ashton *et al.*, 1979; Herning *et al.*, 1981; Hill & Marquardt, 1980; Russell *et al.*, 1975). This is done by increasing the average puff frequency and/or volume or by inhaling more deeply. The probable increase in the intensity with which cigarettes are smoked indicates that the estimated exposure for current smokers is more likely to underestimate than overestimate the true exposure.

Conversely, the average consumption of 29.3 ciga-

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rettes per day is based on sales data for the number of cigarettes sold in 1980 divided by the 1979/1980 National Health Interview Survey estimate of the number of current smokers. Estimates based on interviews with current smokers are not used because smokers significantly underreport their cigarette consumption (Todd, 1978). However, the estimate of 29.3 cigarettes per day may be too high because the 1979/1980 National Health Interview Survey excludes smokers under age 17 and because some of the self-reported never and ex-smokers should be current smokers (NRC, 1986). If the probable increase in smoking intensity is ignored, an increase in the estimated number of current smokers by 10% (to account for smokers under age 17 and misclassified never smokers) decreases the average daily retained exposure from 310 mg to 283 mg for male and from 249 to 228 mg for female current smokers. This is further decreased to 166 mg for male current smokers and to 134 mg for female current smokers by using the lowest experimentally determined particulate retention rate of 47% (First, 1984) instead of an 80% retention rate.

Current Smoker's Average Lung Cancer Rate

The estimated number of never smoker lung cancer deaths increases if the lung cancer risk for current smokers increases. This occurs if the estimated number of lung cancer deaths decreases among (1) ex-smokers, (2) never smokers, and (3) current smokers who die from causes other than smoking. This is calculated for the latter two groups by using the American Cancer Society never smoker lung cancer death rates (Garfinkel, 1981) instead of the rates from the National Mortality Followback Survey. The American Cancer Society rates were not used before because they are for an unrepresentative group of mostly middle-class individuals (Hammond & Seidman, 1980), whereas the National Mortality Followback Survey rates are based on a representative sample of all never smoker lung cancer deaths. The number of ex-smoker lung cancer deaths is minimized if the ex-smoker/smoker mortality ratio from the Veterans study (Rogot & Murray, 1980) is used instead of the average for the American Cancer Society and Veterans studies combined.

These three changes increase the estimated number of current smoker lung cancer deaths from 48,255 to 55,902 male deaths and from 18,394 to 19,954 female deaths. However, this only slightly alters the lung cancer risk because the revised estimate of the average smoker's exposure depends upon increasing the number of smokers by 10%. Given a 10% increase in smokers, the lung cancer rate increases 5.6% to 300 LCD/100,000 male never smokers, but falls 1.7% to 119 LCD/100,000 female never smokers.

The lung cancer death rate for female current smokers is substantially less than the rate for male current

smokers. The difference could partly be caused by women inhaling less deeply than men or by smoking lower tar cigarettes. Alternatively, the lower mortality rate for women could reflect a shorter latency period because women, on average, took up smoking at a later date than men. An upper estimate of the lung cancer death rate for female smokers can be adjusted for this by assuming that the risk/mg of exposure for women is equal to the risk/mg of exposure for men. This estimates a risk of 242 LCD/100,000 female smokers.

Upper Risk Estimate

The upper estimate is 62 lung cancer deaths among never smokers as a result of exposure to particulate ETS. This is based on a lung cancer death rate of 300 LCD/100,000 male and 242 LCD/100,000 female current smokers, an average retained exposure of 166 mg/day for male and 134 mg/day for female current smokers, and a retained exposure of 0.14 mg/day for male and 0.06 mg/day for female never smokers.

Comparison with Other Estimates

The linear extrapolation estimate of 12 deaths, or the upper linear extrapolation estimate of 62 never smoker lung cancer deaths, is substantially less than five alternative estimates, ranging from 265 to 3,610 lung cancer deaths, given in Table 9. All of the alternative estimates use the National Health Interview Survey estimate of the number of never smokers ≥ 35 years of age. Three of the alternatives are risk-based estimates derived from the results of epidemiological studies: one from the difference in lung cancer mortality rates between Seventh-Day-Adventists and other never smokers (termed a phenomenological estimate by the authors), and the other two apply average geometric mean lung cancer risks observed in epidemiological studies to the population-attributable risk equation (Cole & MacMahon, 1971). The two remaining estimates are exposure-based estimates derived from smoker/never smoker exposure ratios and linear extrapolation.

To a certain extent, differences among the various estimates are expected because each type of estimate (for example, linear extrapolation or population-attributable risk methods) requires different assumptions and all estimates are only crude approximations without confidence limits. However, even if the confidence limits for all estimates include all other estimates, this does not explain the 22- to 301-fold difference between the linear extrapolation estimate of 12 lung cancer deaths and the other estimates.

The previous discussion shows that the substantial difference between our estimates and the alternative estimates cannot be adequately explained by errors in our estimates of the number of never smokers, the average exposure for current and never smokers, or

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Table 9. Alternative estimates of the number of ETS-attributable never smoker lung cancer deaths (LCD) (both sexes combined) in 1980 in the U.S.

Method	Description	Estimated LCDs
Phenomenological	Repace and Lowrey's (1985) sex and age-specific difference in LCD rates between Seventh-Day-Adventist (SDA) and non-SDA never smokers is applied to the 1979/1980 National Health Interview Survey estimate of the U.S. population of never smokers by sex and age (Arundel <i>et al.</i> , 1986).	3,610
Linear Extrapolation: Based on ETS Exposure	Repace and Lowrey's (1985) estimate of 555 nonsmoker (never and ex-smokers combined) LCDs is adjusted for the National Health Interview Survey estimate of the percentage (63.87%) of nonsmokers that are never smokers.	354
Linear Extrapolation: Based on Cotinine Ratios	The estimated smoking-attributable LCD rate for current smokers of 284 LCD/100,000 men and 121 LCD/100,000 women is divided by the ratio of the weighted average urine cotinine level of 1483.7 ng/ml for smokers and 5.72 ng/ml for never smokers in four studies (Williams <i>et al.</i> , 1979; Kyrematen <i>et al.</i> , 1982; Jarvis <i>et al.</i> , 1984; Wald <i>et al.</i> , 1984).	265
Population Attributable Risk (PAR) Estimates*		
RR is 1.35	RR is Wald's <i>et al.</i> , (1986) estimate of the geometric mean risk observed in 13 epidemiological studies from six countries. The risk is 1.53 for never smokers living with smokers and 1.18 for never smokers not living with smokers, after adjustment for the misclassification of 7% of ever smokers as never smokers and a relative difference in ETS exposure of 3.0 (Wald & Ritchie, 1984) for the two groups of never smokers. <i>p</i> is 0.249 for male and 0.505 for female never smokers (see text). The average PAR is 0.21 for male and 0.26 for female never smokers.	1,852
RR is 1.14	RR is the geometric mean risk observed in 5 epidemiological studies from the U.S. only (NRC, 1986). The risk is 1.16 for never smokers living with a smoker and 1.05 for never smokers living with never smokers after adjustment for a misclassification rate of 6%, a risk for misclassified ever smokers of 2.0 and a threshold exposure difference for the two groups of never smokers. <i>p</i> is 0.249 for male and 0.505 for female never smokers. The average PAR is 0.072 for male and 0.094 for female never smokers.	655
*The adjusted PAR equation (Eq. 5, p. 292, NRC, 1986) equals:		
$\frac{p_1(RR_1 - 1) + (1 - p_1)(RR_2 - 1)}{p_1(RR_1) + (1 - p_1)(RR_2)}$		
where p_1 is the proportion of never smokers who live with an ever smoker, RR_1 is the risk for never smokers who live with an ever smoker and RR_2 is the risk for never smokers who live with never smokers. The PAR $\times N$, the total number of never smoker LCDs in 1980 (3,479 male, 4,312 female (Table 8)), gives the estimated number of never smoker lung cancer deaths from ETS exposure. The estimates are calculated for each sex and then summed.		

the lung cancer risk for current smokers. On the contrary, there is also evidence to indicate that the estimate of 12 lung cancer deaths is too high. The difference cannot be due to an error in the estimated number of never smokers because all estimates are based on the National Health Interview Survey estimate of the population of never smokers ≥ 35 years of age. There are two possible explanations for the difference: either the alternative estimates given in Table 9 overestimate the true risk, or there are major problems with one or

more of the four major assumptions underlying the linear extrapolation estimate, which causes it to underestimate the true risk.

Alternative Estimates

Several features of the two alternative linear estimates indicate that they could overestimate the true risk. The estimate based on cotinine ratios assumes that cotinine levels in the blood or urine of smokers

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and nonsmokers measure comparable exposures. As discussed earlier, this assumption is probably not valid. Cotinine in smokers measures the lung's exposure to particulate tobacco smoke, whereas cotinine in nonsmokers measures nasal and pharyngeal exposure to the gas phase. Neither the site nor type of exposure is comparable. Repace and Lowrey's (1985) linear extrapolation estimate could also overestimate the true risk from ETS exposure because it appears to overestimate both the never smoker's average exposure and the average smoker's lung cancer risk. The method also uses the inhaled exposure instead of the retained exposure.

The alternative risk-based estimates depend on epidemiological estimates of the lung cancer risk from ETS exposure. The phenomenological estimate is based on a risk of 1.73 for male and 2.54 for female never smokers, calculated from the difference in lung cancer mortality rates for Seventh-Day-Adventist nonsmokers and another group of nonsmokers. The method assumes that the Seventh-Day-Adventists received less exposure to ETS than the other group of nonsmokers. However, the mortality rates for the Seventh-Day-Adventists are based on only 15 female and 10 male deaths. Consequently, these rates are unstable, with large fluctuations in the death rates instead of a consistent increase with each successive age group. These fluctuations could lead to an overestimate of the lung cancer risk from ETS exposure (Arundel *et al.*, 1986).

The population-attributable risk estimates are based on only three parameters: the geometric mean lung cancer risk from ETS exposure, the proportion of never smokers exposed to ETS, and the number of never smokers at risk. The difference between the population-attributable risk and linear estimates cannot be attributed to errors in the estimated value of the number of never smokers at risk because this parameter is also used in the linear estimate. Both methods also assume that all never smokers are exposed to ETS, though the population-attributable risk method calculates separate risks for the proportion of never smokers exposed to ETS at home and the proportion not exposed at home. These proportions are derived from the 1970 National Health Interview Survey estimate that 24.9% of male and 50.5% of female never smokers age 35 and over live with a current or former smoker.

The population-attributable risk method, based on a geometric mean risk of 1.35 from 13 epidemiological studies, estimates 1,852 lung cancer deaths in 1980 among never smokers from exposure to ETS. The method is similar to that used by Robins (1986). Robins' method estimates 1,807 lung cancer deaths in 1980 among never smokers of both sexes from ETS exposure.

The difference in the linear and population-

attributable risk estimates could occur if all estimates of the geometric mean risk overestimate the true risk. Lee (1986) has shown that the misclassification of ever smokers as never smokers could result in an overestimate of the geometric mean risk. However, the population-attributable risk estimates are adjusted for misclassification, though the misclassification rate and the lung cancer risk for misclassified ever smokers is only crudely estimated. The geometric mean risk could also be biased by response or recall bias in the case-control studies on which it is based or by differences in the age distribution of lung cancer cases in the epidemiological studies and the age distribution of never smokers in the U.S. Furthermore, the geometric mean risk is a crude risk; it is not adjusted for age or any other possible or established risk factor for lung cancer such as occupation, socioeconomic status, or diet. The lack of adjustment for other risk factors could bias the geometric mean risk either upwards or downwards.

Assumptions of the Linear Estimate

Three of the four major assumptions of the linear extrapolation estimate can provide a plausible explanation of the difference between our estimate and the population-attributable risk estimates. The assumption of no low threshold, where the risk falls to zero, is irrelevant. The second assumption is that the dose-response relationship is linear. The difference between the estimates can be explained if the true dose-response relationship is nonlinear and convex, such that one unit of exposure at low doses is substantially more carcinogenic than one unit of exposure at high doses. However, most dose-response studies of chemical carcinogens have found sublinear or "hockey stick" shaped relationships between exposure and risk in which one unit of exposure at low doses results in a smaller increase in risk than one unit of exposure at high doses (Hoel *et al.*, 1983). The linear assumption is usually recommended as a conservative estimate of risk because it is believed to err towards overestimating the true risk (Anderson, 1983).

The best fitting equation for the lung cancer death rate per unit of exposure in a 1951-1971 cohort study of 34,440 British doctors is nonlinear and equal to $0.26(\text{dose}+6)^2$, where dose is the average number of British cigarettes smoked at the time of the study (Doll & Peto, 1978). This equation indicates that the linear assumption overestimates the risk from ETS exposure. For example, the sales-weighted average British cigarette between 1951 and 1971 delivered 29.9 mg of particulate tobacco smoke to the smoker (Wald *et al.*, 1981). An average inhaled exposure for male never smokers of 0.62 mg/day of particulate ETS (equivalent to 0.02 British cigarettes) results in an annual excess inhaled-exposure risk of 0.06 LCD/100,000 never

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Table 10. Relative particulate tobacco smoke exposure for current and never smokers in 1955, 1968 and 1980

	1955	1968	1980
Proportion of population that smoke ^a	0.376	0.386	0.345
Tobacco weight of an average cigarette (grams) ^b	1.12	0.95	0.80
Tar (including nicotine) delivery per cigarette (mg) ^c	40.1	23.1	14.2
Cigarettes/day (CPD) smoked by the average smoker ^d	26.2	29.7	29.3
Never smoker's exposure index ^e	11.0	10.9	8.1
Smoker's daily exposure (mg) ^f	1050.6	686.1	416.1
Ratio of smoker's exposure/never smoker's exposure index	95.5	62.9	51.4

^a1955: \geq age 18 (SG, 1979), 1968: \geq 17 (SG, 1979), 1980: \geq 17 (1979/1980 National Health Interview Survey).

^b(USDA, 1985).

^c1955 and 1968 (SG, 1981), 1980 (Tobacco Inst., 1981).

^dFor 1955 and 1968, equals per capita (\geq 18) number of cigarettes sold (Table 1-1, NRC, 1986)/proportion of population who smoke/365. For 1980, equals the number of cigarettes sold/National Health Interview Survey estimate of the number of never smokers \geq 17.

^eProportion of population that smoke \times tobacco weight \times CPD per smoker.

^fTar delivery per cigarette \times CPD.

smokers. Adjusting for the retained exposure decreases the risk to 0.008 LCD/100,000, which predicts 1.4 lung cancer deaths among male never smokers compared to the linear estimate of 8 deaths. The same relative decline for female never smokers would estimate 0.9 female lung cancer deaths, for a combined minimum estimate of 2.3 lung cancer deaths in 1980.

The other two assumptions of the linear estimate (equal risk per unit of exposure for smokers and never smokers, and that all risk is attributable to particulate exposure) can be examined together. The difference between our estimate and the population-attributable risk estimates can be explained if ETS is substantially more carcinogenic per unit of exposure than mainstream smoke. The lowest alternative estimate of 265 lung cancer deaths requires ETS to be as much as 22 times more carcinogenic than mainstream smoke, the highest population-attributable risk estimate of 1,852 lung cancer deaths requires ETS to be as much as 154 times more carcinogenic, and the phenomenological estimate requires ETS to be as much as 301 times more carcinogenic. ETS could be more carcinogenic than mainstream smoke if there were substantial differences in the chemical composition, deposition pattern, or deposition site of passively inhaled sidestream smoke versus actively inhaled mainstream smoke.

The linear estimate is essentially based on a difference in exposure between never and current smokers, while the phenomenological and population-attributable risk estimates are based on a difference in risk between never smokers with and without regular exposure to ETS. Given the large difference in the exposure of never and current smokers, the relative risks of

2.54 and 1.73, on which the phenomenological model is based, or the relative risks of 1.34 or 1.14 used in the population-attributable risk estimates, are far too high. In our opinion, a substantially greater carcinogenicity for ETS versus mainstream smoke is the most plausible factor which could explain the large difference between our linear estimate and the risk-based estimates, assuming that the latter estimates more closely approximate the true risk. A supralinear relationship between exposure and risk could also explain the difference, though this appears less probable. Otherwise, the risk-based estimates must substantially overestimate the true risk. Further research on the relative carcinogenicity of mainstream and sidestream smoke and the dose-response relationship for low exposures to tobacco smoke is necessary.

Effect of Past Exposures

One final problem needs to be addressed. The use of the average particulate ETS exposure for never smokers in 1980 will underestimate the average never smoker's risk if the smoker/never smoker exposure ratio was less in the past than in 1980, and if past exposures are more important in the development of lung cancer than recent exposures. The latter condition may not be true: the lung cancer risk for ex-smokers declines with the number of years since smoking ceased (SG, 1979). The former condition can be examined by estimating the change in exposure over time. The average smoker's past exposure can be determined from the average cigarette consumption and the average particulate delivery. Though the average

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never smoker's past exposure cannot be estimated directly, it is possible to construct a relative exposure index from annual data on the average amount of tobacco in a cigarette, the percentage of the population that smokes, and the average cigarettes per day smoked per smoker.

Table 10 gives the average smoker's exposure as well as the average never smoker's exposure index for 1955, 1968, and 1980. The smoker's exposure has declined 60.4% since 1955 (mostly due to increased filter use), whereas the never smoker's exposure index has declined only 26.4%. The faster decline for smokers means that the 1980 smoker/never smoker exposure ratio is less than the ratio in 1955 (Table 10) and that the 1980 risk estimate for never smokers should overestimate the true risk based on a composite of past exposures.

The estimated average particulate ETS exposure for current smokers in 1980, based on machine-smoked particulate ETS deliveries, would overestimate the decline in the average smoker's exposure if each cigarette is smoked more intensely in 1980 than in 1955. However, the average smoker's exposure will decline faster than the never smoker's average exposure unless the average particulate ETS delivery/cigarette in 1980 is increased by 86% to 26.4 mg. An 86% average increase in the intensity with which each cigarette is smoked is unlikely; experimental studies of smokers given cigarettes with substantially lower tar and nicotine deliveries than their usual brand find the intensity of smoking to increase by 33% to 66% (Herning *et al.*, 1981; Ashton *et al.*, 1979).

Acknowledgements — We thank Ted Irwin for his assistance with the data analysis, Jon Steeves and Mary Weinkam for conducting extensive computer analyses, and Mary Hehn for her assistance with the manuscript preparation.

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Appendix: Equations and Parameters of the Linear Extrapolation Estimate

Never Smoker's Retained Particulate ETS Exposure:

$$\text{Exp N} = \text{NRR}(\text{ExpH} + \text{ExpR} + \text{ExpW} + \text{ExpO})$$

where:

$$\text{Home Exposure (ExpH)} = R \times L_H \times P_H \times A_H$$

$$\text{Restaurant/Bar Exposure (ExpR)} = R \times L_R \times P_R \times A_R$$

$$\text{Work Exposure (ExpW)} = R \times L_W(P_{WW} \times A_{WW} + P_{BW} \times A_{BW} + P_{RW} \times A_{RW})$$

$$\text{Other Exposure (ExpO)} = R \times L_O \times P_O \times A_O$$

R = the respiration rate, L_x = the length of exposure in each location x , P_x = the proportion of never smokers exposed to particulate ETS in each location x , A_x = the ambient particulate ETS level in each location x , WW = white-collar workplace, BW = blue-collar workplace, NRR = the never smoker particulate ETS retention rate.

Smoker's Retained Particulate Tobacco Smoke Exposure:

$$\text{ExpSM} = \text{SMRR}(\text{CPD} \times \text{TAR})$$

SMRR = the smoker particulate tobacco smoke retention rate, CPD = the average number of cigarettes smoked/day/smoker, TAR = the average tar delivery/cigarette.

$$\text{Smoker's Lung Cancer Death (LCD) Rate: SMLCDR} = 100,000(\text{NSMLCD}/\text{SMPOP})$$

where:

$$\text{NSMLCD} = \text{TLCD} - \text{LCDN} - \text{LCDEX} - \text{LCDSM}$$

NSMLCD = the number of smoking-attributable LCDs among current smokers, SMPOP = the total number of current smokers, TLCD = the total number of LCDs in 1980, LCDN = number of LCDs which occurred among never smokers, LCDEX = number of LCDs which occurred among ex-smokers, LCDSM = number of LCDs which occurred among smokers from nonsmoking causes.

where:

$$\text{LCDEX} = (\text{TLCD} - \text{LCDN})(1 + \text{NCS}/\text{NES} \times 2.26)$$

where:

NCS = the number of current smokers, NES = the number of ex-smokers.

Never Smoker's LCD Rate: NLCDR =

$$\text{SMLCDR}(\text{ExpSM}/\text{ExpN})$$

SMLCDR = the LCD rate per 100,000 smokers, ExpSM = average smoker's particulate tobacco smoke exposure, ExpN = average never smoker's particulate ETS exposure.

$$\text{Number of Never Smoker Lung Cancer Deaths from ETS Exposure} = (\text{NPOP}/100,000)\text{NLCDR}$$

NPOP = number of never smokers, NLCDR = the lung cancer death rate per 100,000 never smokers.

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Indoor Air Quality

Symposium

December 6 - 7 , 1988

**San Carlos de Bariloche
Argentina**


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BIOLOGICAL EFFECTS AFTER EXPOSURE TO ETS

by *FRANZ ADLKOEFER*

Prof. Dr. med. Franz Adlkofer, born in 1935, studied medicine at Munich University. In 1963 he obtained the degree of Doctor of Medicine at the Max-Planck-Institute of Biochemistry in Munich. From 1960 to 1976 he worked at Klinikum Steglitz, Free University of Berlin and was acknowledged as a specialist in internal medicine. In 1974 he spent a sabbatical year at the Nuffield Department of Biochemistry at Oxford University, U.K. In 1980 he was appointed Professor of Internal Medicine at the Free University of Berlin. In 1976 he became Director of the Scientific Department at the Verband der Cigaretten Industrie, Hamburg, and at the same time Scientific Secretary to the Research Council on Smoking and Health, Hamburg. He has held these posts since then. He lectures on environmental toxicology at the Free University of Berlin.

The 'passive smoking' issue has given rise to much public controversy in both the USA and in Europe. This is mostly due to the fact that the present state of knowledge does not permit a precise risk evaluation of passive smoking. In my view, however, it is sufficient to give an idea of the extent of the problem.

I do not intend to deal with the annoying effects of passive smoking - they are beyond doubt - nor will I concentrate on the question of whether passive smoking may be involved in the development of lung disease and coronary heart disease, as has been suggested on some occasions. My intention is to investigate the hypothesis that passive smoking may play a role in the development of cancer.

The following conclusions were drawn by an IARC Working Group on

the Evaluation of the Carcinogenic Risk of Chemicals in Humans at their meeting in Lyon in February 1985 (1):

"The observations on non-smokers that have been made so far are compatible with either an increased risk from 'passive' smoking or an absence of risk. Knowledge of the nature of sidestream and mainstream smoke, of the materials absorbed during 'passive' smoking, and of the quantitative relationship between dose and effect that are commonly observed from exposure to carcinogens, however, leads to the conclusion that passive smoking gives rise to some risk of cancer."

This statement reflects the uncertainty of scientists who have given serious thought to the issue of passive smoking. In the light of this uncertainty it is not surprising that there are conflicting views on the subject. On the one hand, according to a recent statement by a well-known German toxicologist, tobacco smoke is the most dangerous mixture of toxic compounds for man in the indoor environment (2). On the other hand, this issue is not considered relevant enough to justify spending time or money on its solution. This conclusion may be drawn from an analysis of passive smoking which another well-known German toxicologist published only a few days ago (3).

The following comments will concentrate on the effects of passive smoking from a toxicological point of view. In this connection we should examine to what extent current toxicological data confirms the view of the Lyon working group that, irrespective of the epidemiological data, it is more plausible to assume a slightly elevated risk from passive smoking than the alternative, namely that there is no risk at all. The logic behind the argument that a risk exists is simple, if not simplistic.

- If smoking causes cancer and other diseases, the same would apply to passive smoking; after all there is no doubt that ETS is tobacco smoke as well.
- Tobacco smoke contains genotoxic, i.e. carcinogenic substances; therefore their mere presence is assumed to indicate risk, because no threshold values have been accepted for such substances so far.

Before dealing with these points, which are the key arguments put forward in this debate, I would like to define some of the terms necessary for an understanding of the factors involved (4).

- Mainstream smoke (MS) is produced at the burning end of the cigarette at temperatures of about 800-900 °C, drawn through the cigarette and inhaled by the smoker.
- Sidestream smoke (SS) is formed between puffs at the burning end of the cigarette at a temperature of 600 °C.
- Environmental tobacco smoke (ETS) is a mixture of approximately 15-20% of the exhaled mainstream smoke and 80-85% of sidestream smoke.

1. EXTRAPOLATION OF THE RISK INVOLVED IN PASSIVE SMOKING ON THE BASIS OF THE RISK DUE TO SMOKING

Tobacco smoke can be divided into the gaseous phase and particulate matter. Some of the main substances present in the gaseous phase of mainstream and sidestream smoke and of course in smoke-polluted air are listed in Table 1. Except for nicotine, which is tobacco-specific, all these substances are commonly found in the environment and originate from sources other than tobacco smoke. The same is true of benzene and volatile nitrosamines, which are carcinogenic compounds. The concentration of these toxic substances increases more or less in proportion to the number of cigarettes, cigars or pipes smoked and in relation to the room dimensions and its ventilation (4,5).

Table 2 shows substances or groups of substances present in the gaseous phase of mainstream and sidestream smoke as well as in smoky room air. These include, for example, the polycyclic aromatic hydrocarbons (PAH) which are normally found in the environment -the best known of these is BaP- and tobacco-specific nitrosamines (TSNA). The latter are found only in tobacco smoke. Animal experiments have shown that PaH and TSNA are definite carcinogens. As can be seen from Table 2, there is no doubt that ETS contains toxic substances some of which are mutagenic and carcinogenic: these are inhaled with smoke-polluted air (4,5).

The question inevitably arises whether the amounts of substances inhaled by passive smoking are sufficient to cause disease. Table 3 shows the amounts calculated for some of the main volatile and particle-bound substances and groups of substances. The column on the left gives an estimate of the amount of these substances deposited in the respiratory tract by smok-

Table 1
Substances of importance in the gaseous phase of mainstream smoke (MS) and sidestream smoke of cigarettes (SS) and in room air under realistic conditions.

	MS (range/cigarette)		SS		Concentration range in room air	
Carbon monoxide (CO)	2 - 20	mg	46 - 61	mg	3 - 20	ppm
Nitrogen oxide (NO)	0.07 - 0.17	mg	1.6 - 3	mg	50 - 200	ppb
Nitrogen dioxide (NO ₂)	n.n.		0.16	mg	10 - 70	ppb
Ammonia (NH ₃)	50	μg	5,300 - 8,500	μg	100 - 450	μg/m ³
Hydrogen cyanide (HCN)	150 - 550	μg	100 - 250	μg	10 - 120	μg/m ³
Formaldehyde	20 - 90	μg	450 - 1,500	μg	20 - 100	μg/m ³
Acetic aldehyde	18 - 1,400	μg	2,400	μg	400 - 500	μg/m ³
Acrolein	25 - 140	μg	925	μg	15 - 25	μg/m ³
Nicotine*	[0.5 - 2	mg]	3 - 4	mg	20 - 100	μg/m ³
Benzene	10 - 100	μg	488	μg	5 - 16	μg/m ³
Volatile Nitrosamines:						
NDMA	0.2 - 20	ng	155 - 398	ng	5 - 70	ng/m ³
NPYR	2.4 - 29	ng	7 - 150	ng	1 - 5	ng/m ³

*Nicotine is a constituent of particulate matter in mainstream smoke.

Table 2
Substances of importance in the particulate matter of mainstream smoke (MS) and sidestream smoke of cigarettes (SS) and in room air under realistic conditions

	MS (range/cigarette)		SS		Concentration range in room air	
Particles (TPM)	5 - 30	mg	20 - 50	mg	0.1 - 0.5	mg/m ³
Nicotine*	0.5 - 2	mg	[3 - 4	mg]	[20 - 100	μg/m ³]
Phenol	10 - 130	μg	270 - 320	μg	< 1 - 20	μg/m ³
Tobacco-specific Nitrosamines:						
NNN	0.2 - 5.5	μg	0.15 - 6	μg	< 1 - 6	ng/m ³
NNK	0.1 - 4.2	μg	0.2 - 0.8	μg	< 2 - 11	ng/m ³
Benzo(a) pyrene	10 - 50	ng	25 - 103	ng	3 - 25	ng/m ³
Cadmium	100	ng	430 - 720	ng	9 - 31	ng/m ³

*Nicotine is a constituent of the gaseous phase in sidestream smoke and in room air.

ing 20 cigarettes, i.e. the average daily consumption. An estimated deposition rate for the same substances taken up into the lungs during an 8-hour exposure to tobacco smoke is shown in the middle column. The right column gives the factor indicating how much more of these substances has been taken up by active smoking as compared to passive smoking. What is striking is that the amount of gaseous phase substances inhaled by the smoker is certainly much higher than that taken up by the passive smoker. The fact that the smoker himself is the heaviest passive smoker has not been taken into account in this estimate. Notwithstanding this fact, the difference is less than one order of magnitude. With regard to the particle phase there is a much greater difference. The amount of particulate matter inhaled during smoking is up to 1000 times the amount taken up from passive smoking. It should be noted here that our calculations are in line with those of American authors (6). According to their estimates, the average daily uptake of particles amounts to 310 mg for male smokers while male passive smokers take up less than one in four thousand of this amount. In female smokers the respective figures are somewhat lower, but the factors remain unchanged.

As we have already seen from Table 3, considerable problems arise when one tries to extrapolate the risk of passive smoking on the basis of the health risk associated with smoking because the uptake of toxic substances is different in each case. The following considerations clearly demonstrate that it is not permissible to base calculations on such comparisons.

- The ratios of the substances in sidestream and mainstream smoke vary from substance to substance by factors between 0.5 and > 100 (4,5). Consequently, the smoke mixtures vary in quality.
- ETS mainly consists of sidestream smoke and is almost always mixed with substances from sources other than tobacco smoke (6,7).
- The toxic effect of ETS also decreases due to ageing and is much less strong than that of fresh mainstream smoke inhaled by the smoker (8). It is not yet known whether the same applies to its carcinogenic effect, but this seems probable.
- There are considerable differences between passive and active smoking with regard to the breathing pattern which determines the inhalation depth and lung deposition rate for constituents of particulate matter and the gaseous phase. Passive smoking occurs mainly via nasal

breathing which filters off at least some of the toxic substances found in relatively low concentrations in ETS.

- Only some 10% of ETS particulate constituents are retained in the respiratory tract during passive smoking, whereas 45-95% of particulate matter of mainstream smoke remain in the lung during active smoking (9,10).
- Non-smokers who are frequently or occasionally exposed to tobacco smoke normally have well-functioning clearing mechanisms of the respiratory tract. This means that the particulate matter is partly removed from the lung before it causes any damage.
- It is unlikely that the well-known induction of enzymes which transform procarcinogens into carcinogens in smokers (11) could be found in passive smokers. Whether this enzyme induction has any harmful, or indeed beneficial, effects to the individual has not yet been determined. In any case, the initial conditions with regard to carcinogenesis are not comparable.
- The concentrations of toxic substances taken up by passive smoking are rather low as compared with active smoking. Here, the basic problem of risk assessment at the lowest doses arises, a problem which has not been solved to date(12).

Of course, no one really knows whether this risk curve (Fig. 1) develops in a linear direction, as is generally assumed in the risk evaluation procedure, or whether it takes an S-shaped, i.e. a sublinear course in the lower part of this graph, or whether the course of the curve determines the extent of the risk. The fact that there are numerous defense mechanisms against carcinogenic substances in humans supports the assumption that the dose-effect-relationship curve takes a sublinear course. This is in line with the findings obtained in man and in animals.

2. EFFECT OF PASSIVE SMOKING ON THE HUMAN ORGANISM

Having dealt with the more theoretical aspects of the issue, we should consider the findings on the measurable effects of passive smoking on the human organism published so far. Exposure to tobacco smoke can be demonstrated, for instance, by determining COHb, i.e. hemoglobin-bound

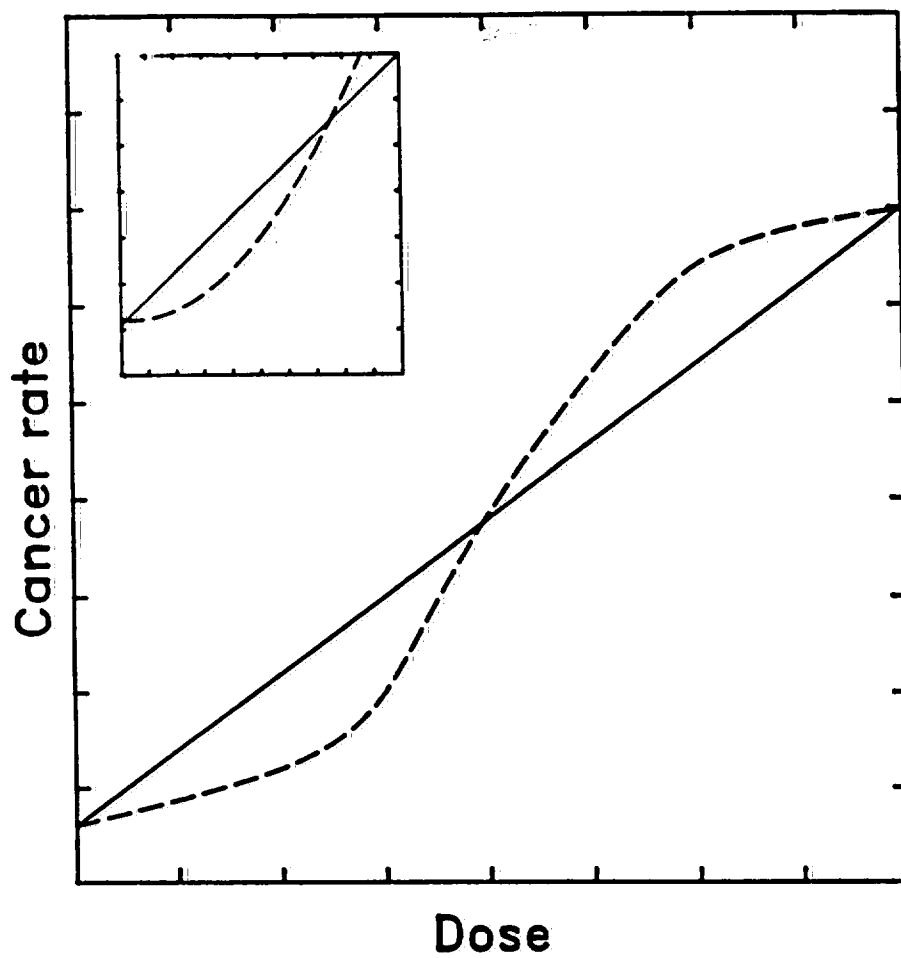


Figure 1.

Possible dose response curves in carcinogenesis

carbon monoxide which occurs at low concentrations under physiological conditions. COHb normally rises only slightly in passive smokers so that it is difficult to differentiate from COHb values measured in non-smokers. Only at extremely high exposure levels, which are unlikely to occur under real-life conditions, COHb can rise by up to 2% (13, 14).

The significance of COHb values is limited by the fact that carbon monoxide, which originates from sources other than tobacco smoke, occurs nearly ubiquitously and might thus simulate a burden due to tobacco smoke. A much better way of demonstrating exposure to tobacco smoke is the determination of nicotine or cotinine in body fluids. Cotinine is the main metabolite of nicotine and unlike nicotine remains metabolically inactive in the body for a longer period. The findings of Jarvis and Russell from England (15,16) may serve as examples here (Tab.4). The authors interviewed out-patients about their tobacco smoke exposure, and the answers were subdivided into three groups headed "Smokers, Passive Smokers and Non-smokers". As for nicotine, marked differences between non-smokers and passive smokers can only be found by measuring it in the urine. In smokers the nicotine concentration is 100 times that measured in passive smokers. A far better indicator than nicotine is cotinine with its long half-life. Again the measurements obtained in urine appear to be the most convincing. Cotinine measured in smokers is again more than 100 times the values found in non-smokers. However, these data should not lead us to conclude that the amount of tobacco smoke passive smokers take up on the average is one hundredth of the amount taken up by smokers. Such a conclusion presupposes that mainstream and sidestream smoke and of course ETS are comparable in their compositions, at least to a certain extent. But this is not the case as has been shown before. This assumption is furthermore limited by the fact that the physico-chemical properties of nicotine vary in mainstream and sidestream smoke and in the room air as well (17).

Now I would like to present some results of our own obtained in studies of non-smokers exposed to tobacco smoke under controlled conditions (18,19).

The day after their admission to the laboratory the subjects stayed in a closed room from 8 am to 4 pm. Smoking was not allowed. The following day the subjects stayed in that room for the same period. This time cigarettes were smoked so that CO rose to a level of 10 ppm in the first experiment and 25 ppm in the second.

Table 3
Estimated uptake of tobacco smoke constituents by active and passive smoking

Tobacco Smoke Constituents		Smoking (20 cig/day)	Passive Smoking (8h/day)*	Range (Smoking/Passive Smoking)
Gaseous Phase:				
CO	(mg)	40 - 400	14.4 - 96	2.7 - 4.2
Nicotine	(mg) ¹	10 - 40	0.08 - 0.4	100 - 125
Formaldehyde	(mg)	0.4 - 1.8	0.08 - 0.4	4 - 5
Volatile Nitrosamines	(μg)	0.05 - 1.0	0.03 - 0.4	1.5 - 2.5
Particulate Matter				
Particles	(mg)	100 - 400	0.024 - 0.24**	1,700 - 4,000
Tobacco-specific Nitrosamines	(μg)	6 - 60	0.002 - 0.010**	3,000 - 6,000
Benzo(a) pyrene	(μg)	0.2 - 1.0	0.001 - 0.011**	90 - 200
Cadmium	(μg)	2	0.001 - 0.014**	143 - 2,000

¹ Nicotine is part of particulate matter in mainstream smoke.

* Assumed breathing volume: 0.5 m³/h

** Assumed lung deposition rate: 11 %

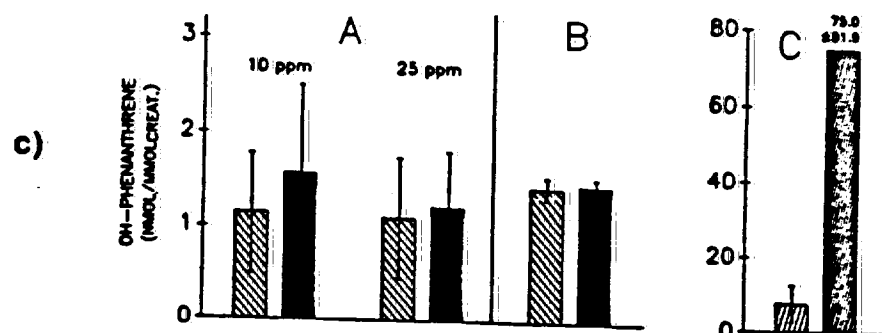
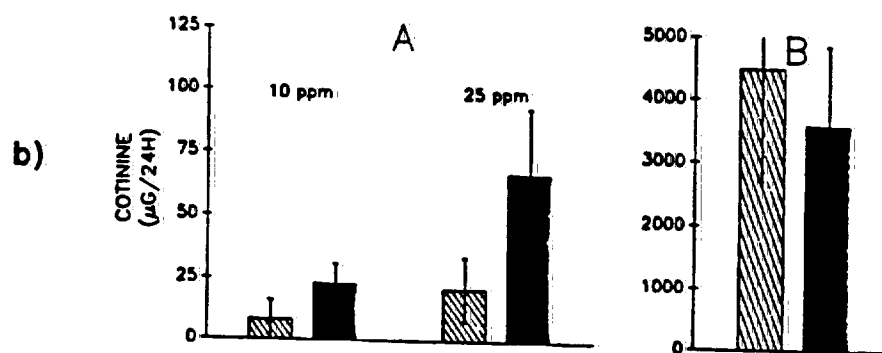
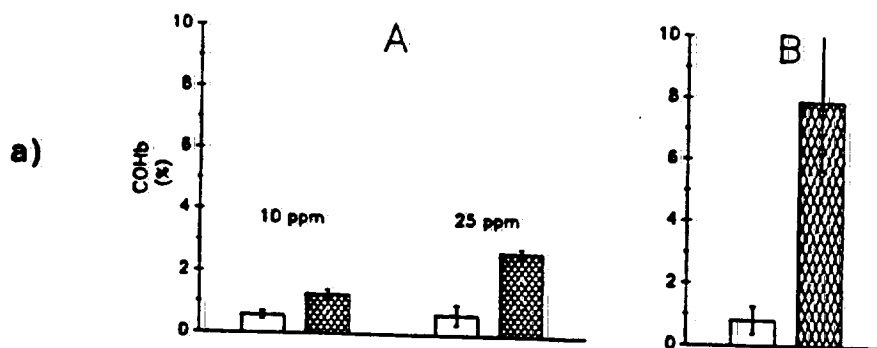
Table 4
Determination of nicotine and cotinine in bodyfluids of non-smokers exposed and not exposed to tobacco smoke and of smokers

Group	N	Substance (ng/ml)
Non-smokers not exposed	46	Nicotine in plasma: 1.04
Non-smokers exposed	54	Nicotine in plasma: 0.77
Smokers	94	Nicotine in plasma: 14.80
Non-smokers not exposed	46	Nicotine in urine : 3.87
Non-smokers exposed	54	Nicotine in urine : 12.11
Smokers	94	Nicotine in urine : 1,749.90
Non-smokers not exposed	46	Cotinine in plasma: 0.82
Non-smokers exposed	54	Cotinine in plasma: 2.04
Smokers	94	Cotinine in plasma: 275.20
Non-smokers not exposed	46	Cotinine in saliva : 0.73
Non-smokers exposed	54	Cotinine in saliva : 2.48
Smokers	94	Cotinine in saliva : 309.90
Non-smokers not exposed	46	Cotinine in urine : 1.55
Non-smokers exposed	54	Cotinine in urine : 7.71
Smokers	94	Cotinine in urine : 1,391.00

Sources:

M. Jarvis et al., J. Epidemiol. Community Health 38, 1984, 335-339;

M.A.H. Russell, Toxicol. Lett. 35, 1987, 9-18.



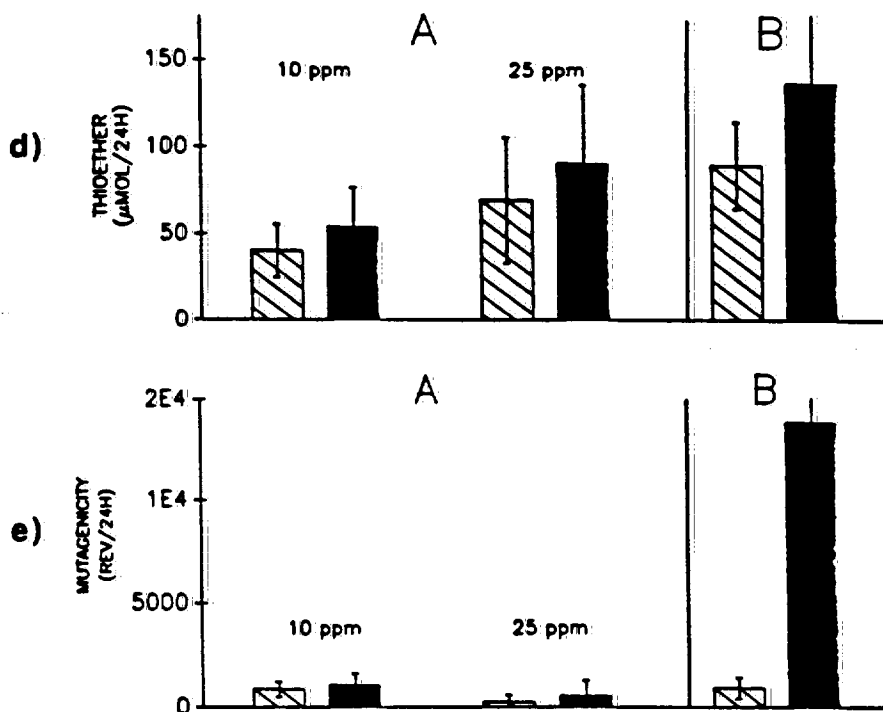


Figure 2:

Effects of ETS exposure for 8 h (A) and cigarette smoking (B) on:

- the carboxyhemoglobin levels in blood before (□) and after (▨) exposure,
- the urinary cotinine excretion on the control (▨) and on the exposure (■) day,
- the urinary hydroxyphenanthrene excretion on the control (▨) and on the exposure (■) day compared to that of road paving workers (C) before (▨) and after (▨) shift,
- the urinary thioether excretion on the control (▨) and on the exposure (■) day,
- the urinary mutagenicity on the control (▨) and on the exposure (■) day.

For more details refer to text.

COHb increased by less than 1% after an 8-hour exposure to the lower concentration of tobacco smoke and by more than 2% after exposure to the higher concentration (Fig. 2a). Such a rise in COHb is of no toxicological relevance for healthy adults. As can be seen from these data, the tobacco smoke exposure exceeded the level found in real-life situations in both experiments, particularly in the second.

As was the case with COHb, the urinary cotinine excretion increased in relation to the dose (Fig. 2b). Even experimental exposure to 25 ppm CO produced cotinine excretion only 1/65th of the levels found in smokers. It is widely accepted that a nicotine uptake which leads to a urinary cotinine excretion as found in our studies is of no relevance from a toxicological point of view.

The urinary hydroxyphenanthrene excretion was not found to increase after exposure to both concentrations (Fig. 2c). Hydroxyphenanthrene is a metabolite of phenanthrene found in tobacco smoke which belongs to the group of PAH. This group of substances is most likely to be involved in the development of lung cancer. The enormously elevated values shown on the right of the graph have been measured in the urine of bitumen-exposed workers asphaltting roads. Significantly, these values are by two orders of magnitude higher, but this is not the subject which concerns us here.

Urinary thioether excretion rose significantly after exposure to both concentrations of tobacco smoke (Fig. 2d). This confirms the assumption that passive smoking leads to the uptake or in-vivo formation of potentially genotoxic substances which have not been identified so far. But there is evidence indicating that they originate for the most part from the gaseous phase. By formation of thioether, toxic substances which the organism takes up from the air and especially from food or which are formed in the organism itself, are detoxified and excreted. Formation of thioether can thus be regarded as an essential protective mechanism of the organism.

No significant rise in urinary mutagenicity was found in the passive smokers of our studies (Fig. 2e). The fact that urinary mutagenicity is elevated in smokers is most probably due to the uptake of particles which, as was said before, play a minor role in passive smoking.

In order to find an answer to the question of whether ETS has a considerable carcinogenic potential, a long-term animal experiment with rats is required. The results of our 90 day pilot-study which has just been terminated cannot yield conclusive evidence (20). The design of the study was similar to

that of the diesel exhaust experiments in which an elevated rate of lung cancer was found in rats and mice. As in the diesel exhaust experiments, the tobacco smoke concentration corresponded to a particle concentration of $4-5 \text{ mg/m}^3$ and was thus far above that normally found in passive smoking. The period of exposure was 10 hours a day, 5 days a week over 90 days. Except for minor changes in the nasal dorsal area of the animals, which were found to recede after the end of the treatment, no histopathological changes could be observed by light microscopy.

The results differed considerably from those obtained with diesel exhaust. Whereas in rats exposed to diesel exhaust for just a few days a considerable deposition of soot is found in the lungs which remains there even 90 days after termination of treatment, this is, surprisingly, not the case with hamsters. Many scientists suggest that this overload of the lungs with diesel soot, which causes permanent irritation thus accelerating cell turn-over rate, is the actual cause of the dose-related development of lung cancer in these animals (21,22).

In the light of the findings obtained so far it is questionable whether an exposure to ETS under conditions similar to those of the diesel exhaust studies leads to a rise in lung cancer rates in rats. These doubts may furthermore be justified in view of the results published by the EPA of the United States suggesting that the carcinogenic and mutagenic potential of cigarette smoke condensate is far lower than that of diesel exhaust (23).

TO SUM UP:

Passive smoking leads to an uptake of minute amounts of mutagenic and carcinogenic substances into the organism. This applies especially to particle-bound substances which are suspected of playing a major role in the development of cancer due to smoking. Compared with the non-smoker's daily total exposure to presumably carcinogenic substances, e.g. at home, at the workplace, in the diet as well as in the endogenous metabolism, the uptake of such compounds with ETS represents only a minor dosage. It is therefore not surprising that no genotoxic effects of passive smoking on the human organism have been demonstrated so far. This means that a risk assessment of passive smoking is not possible at present. Instead of accepting this fact it is claimed that the presence of carcinogenic substances even in minute con-

centrations can lead to irreversible damage of the genetic material. Indeed, the logic of such a scientific approach compels us in line with the IARC Working Group in Lyon to conclude that passive smoking must involve at least a slightly increased risk of cancer. Whether a real risk is involved which in the future can be demonstrated on the basis of measurable results remains an open question. In the light of this health officials should concentrate on more significant environmental problems in the long-term interest of society, rather than wasting time and money on trivial issues.

We as scientists unable to prove or disprove the possibility of a slightly increased risk may find this situation somewhat frustrating, but unfortunately this dilemma will probably accompany us for years to come. Johann Wolfgang von Goethe - Germany's greatest poet - who incidentally was also a scientist of some reputation - expressed this problem in the words of Dr. Faustus:

By night and day a mystery tender,
Nature guards her secrets well:
And what she will not freely render,
She never on the rack will tell.

Goethe, Faust I

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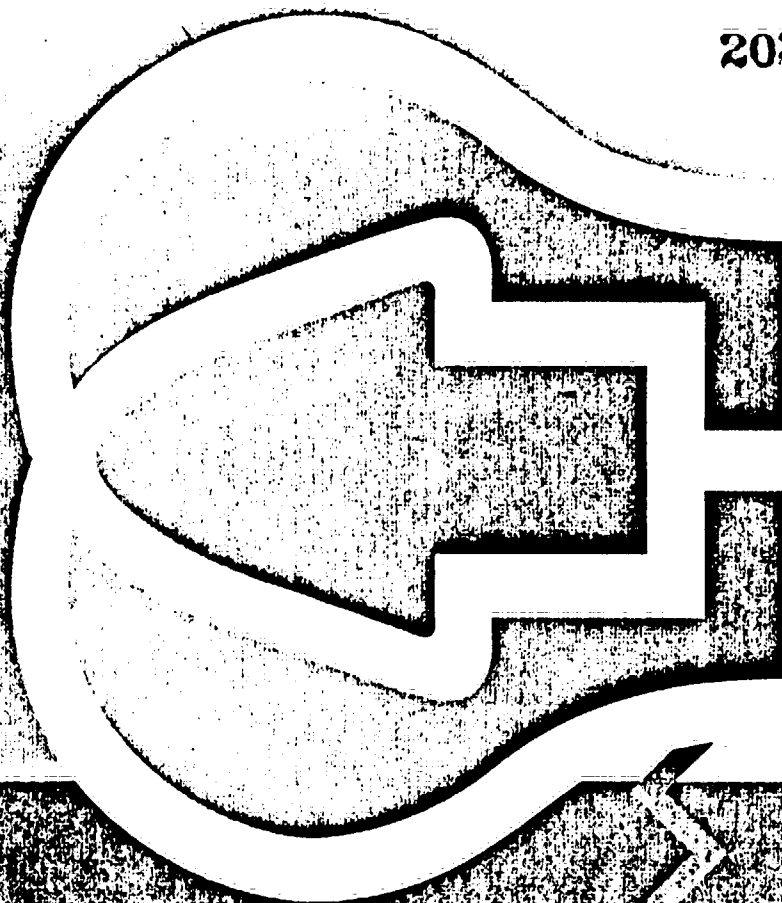
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THE FIFTH
INTERNATIONAL
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ON INDOOR AIR
QUALITY AND
CLIMATE

TORONTO, CANADA
July 29 - August 3
1990



RESPIRATORY DEPOSITION OF
ENVIRONMENTAL TOBACCO SMOKE

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There has been much concern in recent years concerning the health implications of 'passive' or 'secondary' smoking. The physical characteristics, and hence respiratory deposition of mainstream, fresh sidestream, and aged sidestream smokes are markedly different and this must be taken into account when assessing risk. Data obtained from volunteers inhaling radiolabelled smoke in each form is presented showing the dose to the bronchial region of the lung from environmental tobacco smoke (ETS) particles to be very much less than would be expected from epidemiological estimates, although pulmonary particle deposition and vapour deposition are enhanced. This is discussed with reference to the effects of changes in particle size and evaporation / condensation processes on the regional deposition of smoke aerosols.

INTRODUCTION

In recent years, there has been increasing public concern as to potential health risks from environmental tobacco smoke (a combination of aged sidestream and exhaled mainstream smoke). This concern has been focused by publication of three reports concerning exposure to ETS both in the U.K. and the U.S. (1,2,3). It has also been reflected in a number of airlines banning smoking on short-haul flights and, for example, in recent legislation in Canada prohibiting smoking in federally owned buildings. Although many surveys have been carried out using biological indices of smoking in 'passive' smokers, these have reflected exposure to gaseous and vapour components. Exposure to carbon monoxide has been determined by measurement of end-expired CO or carboxyhaemoglobin and to nicotine by measurement of either nicotine or cotinine (a major metabolite) in saliva, plasma or urine. Of these indices, cotinine has been most widely quoted as nicotine can be regarded as the major tobacco-specific product identifiable in the population. It should be noted, however, that these markers can only be used as an indicator of systemic uptake, and cannot be related directly to the regional deposition of tar particulate material.

Models of the estimation of relative risk from exposure to mainstream and sidestream smoke particles must take account of the deposition efficiency of individual components of the smoke and their regional deposition and clearance. This also applies to volatile components generated with the smoke. Physical factors affecting deposition efficiency include evaporation/condensation equilibria, particle concentration and particle size. In this work, we shall describe the results

obtained from radio-tracer studies of the physical behaviour of mainstream and sidestream smoke and their deposition in volunteers. Special attention has been paid to the deposition of tar particulate material in the lung tracheo-bronchial (TB) region, where the majority of lung cancers in humans originate. Vapour deposition will also be considered and the limitations of the model used will be discussed.

MATERIALS & METHODS

Radiotracer

The physical and chemical behaviour of cigarette smoke is extremely difficult to model as several thousand constituent compounds have been identified (4). Thus, of necessity, any tracer compound can only represent an average behaviour. 1-iodohexadecane ($C_{16}H_{33}I$) was chosen as a tracer for the physical behaviour of sidestream smoke as it has a boiling point ($380^{\circ}C$) in the middle of the range of compounds found in tar particulate material. It may be labelled with any radio-isotope of iodine, and has proved to be a useful marker of particulate material in mainstream cigarette smoke (5).

Exposure system

Details of the exposure facility and ETS generation have been described elsewhere (6). In brief, a Mason-BAT smoking machine is used to introduce sidestream smoke into an environmentally controlled 14.4 m^3 chamber. By varying the number of cigarettes smoked, the smoke/air ratio at the inlet, and the chamber ventilation rate, concentrations ranging from 6 - 135 ppm CO and 0.5 - 18.8 mg.m^{-3} for particulate material (PM) were achieved and could be maintained at steady-state for extended periods. The specific activity, that is, the radioactivity per unit mass of tar collected was shown to be consistent over generation periods of up to 60 minutes and was independent of particle size (7). The coefficient of variation for CO and PM concentrations measured at twelve sampling sites within the room was 6.5%, demonstrating good spatial mixing. Regressions for both CO and PM concentrations were calculated versus a generation factor (number of cigarettes burning \times fraction of smoke diverted to room / number of air changes per hour) giving equations:-

$$\begin{aligned}\text{CO concentration} &= 50 \times \text{generation factor} + 5.2 \quad (r^2 = 0.95) \\ \text{PM concentration} &= 7.3 \times \text{generation factor} - 0.56 \quad (r^2 = 0.97)\end{aligned}$$

RESULTS & DISCUSSION

Physical behaviour of smoke

On generating sidestream smoke atmospheres within the chamber it was found that, at equilibrium, when smoke generation ceased, that the half-time of clearance for PM was more rapid than that for CO, implying mechanisms of removal for PM other than dilution by clean air through continuing ventilation. It was also noted that gravimetric yields of PM from the aged sidestream smoke were approximately 30% of those of fresh (<2s old) sidestream smoke collected directly from the burning cigarette via the 'fish-tail'

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sampling hood (8). By radiolabelling with 123I-1-iodohexadecane, it was demonstrated that only a small fraction of the label was found on the walls of the chamber, indicating deposition to surfaces to be a minor mechanism of removal. Sampling of the steady state atmosphere onto glass fibre filters backed by charcoal packs showed up to 70 % of the airborne activity in the room was in the vapour phase, compared to less than 5 % vapour in fresh sidestream smoke. The figure of 70 % vapour is in good agreement with the reduced particulate recovery by mass. Hence, it appears that as sidestream smoke is generated and ages under ambient conditions, dilution occurs causing evaporation of a significant mass fraction of the particles (in approximately the same proportions as the radiolabel). As this phenomenon appears to be a dynamic vapour/particle equilibrium, it will be related to vapour pressure and thus, ambient temperature together with dilution, both of which may affect sampling. This was reflected, experimentally, by an approximately linear increase in the ratio of PM mass to CO concentrations as the amount of smoke generated i.e. the generation factor increased ($r^2 = 0.84$). In contrast, studies measuring the decay of nicotine generated in sidestream smoke in the chamber showed its removal to be faster than other vapour phase components (9), implying deposition on surfaces to be a more significant mechanism of removal.

Mainstream smoke deposition

By using the 123I-1-iodohexadecane label, direct measurements of tar deposition in volunteers can be carried out by gamma-counting, using an array of six collimated sodium iodide crystals described elsewhere (10). Estimates of regional deposition were made by differential lung clearance. This was shown to give a two-phase clearance with half-times of 2 and 17 hours respectively. The fast clearance was shown to have a similar half-time of clearance to radio-labelled 2.5 μ m polystyrene latex microspheres, implying the fast clearance of the 1-iodohexadecane is due to clearance from the TB region. The slower phase will in contrast, be determined in the main, by the rate of dissolution of the label from the particles in the pulmonary (P) region. Measurements were carried out on 23 male volunteers, smoking cigarettes typical of the sales-weighted average tar yield (14 mg) in the U.K., and were found to have an average tar intake of 14.3 mg per cigarette (Range = 6.0 - 24.7 mg), with an average cigarette consumption of 30 cigarettes per day (Range = 9 - 57). It should be noted that the consumption figure in this group is significantly greater than the average for the U.K. Previous differential clearance measurements showed approximately 55% of the tar deposit to be in the TB region with 30% penetrating to the P region, and the remaining 15% in the upper respiratory tract. In addition, exhalation capture techniques have shown, on average, 80% of the tar inhaled to be deposited. The pattern of regional deposition is very different to that predicted by ICRP (11) for particles of 0.8 μ m diameter, as measured in mainstream smoke by cascade impaction (5). This implies that the tar particles, which exist as liquid droplets behave as larger particles within the respiratory tract due either to coagulation at the high particle concentrations found (up to 1010 ml⁻¹),

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hygroscopic growth or a combination of both. Calculations in the measured group of smokers, imply a daily TB deposit of tar of 250 mg/day (Range = 120 - 610 mg).

Sidestream smoke deposition

Unfortunately, no radiotracer measurements could be carried out for deposition of exhaled mainstream smoke, as radiological doses to those generating the smoke could not be justified on ethical grounds. Hence, all values considered are for deposition of aged sidestream smoke, which forms the major component of ETS in any case.

In contrast to mainstream smoke, sidestream smoke particles have had time to age and have been diluted before inhalation. As these processes occur, material evaporates from the particles leaving an involatile core of approximately 0.2 μ m mean diameter at equilibrium. Such particles are likely to behave as predicted by the ICRP model (11). Earlier work assuming an 8 hour workplace exposure to ETS, a particle concentration of 0.9 mg.m⁻³, which is at the high end of reported survey values, and a minute volume of 10 l.min⁻¹ suggests that 4.3 mg of tar particles per day will be inhaled (12). Approximately 10% of this material will be deposited in the lung (13) in the ratio 10% TB : 90% P (11). Thus the daily dose of tar particulate to the TB region of the lung from sidestream smoke will average 0.043 mg, a TB deposition factor of 0.00017 relative to mainstream smokers. Studies of sidestream smoke deposition in volunteers using the 123I-1-iodohexadecane radiolabel are much more difficult to perform than the equivalent mainstream deposition experiments, primarily due to the high proportion (70%) of the label in the vapour phase, which can lead to external contamination problems. In addition, measurements of regional deposition by differential clearance become more difficult to interpret as the label in the vapour phase is transferred more rapidly to blood, giving rise to a higher than normal circulating, background activity. A series of experiments on three volunteers showed an average total deposition of 64% of the label inhaled from aged sidestream smoke at equilibrium. When inhalation experiments were carried out for the vapour phase alone, 87% of the inhaled material was retained, mainly in the upper airways external to the lung. If this figure of 87% deposition is assumed for the deposition of ETS vapour (70% by mass), it implies that 61/64 % of total deposition measured is due to vapour. The remaining 3% of total deposition deriving from particulate ETS (30% by mass) implies a deposition efficiency for the particulate of approximately 10%, which is in good agreement with the figure derived experimentally by Hiller (13) and used in the above calculations. It should be noted, however, that the deposition efficiency figure of 10% derived from our experiments is prone to large variation due to its method of calculation (subtracting one large number from another).

Thus it can be seen that, although the TB dose of tar in 'passive' smokers is only 0.02% of that observed in mainstream smokers, relatively higher doses of smoking products occur elsewhere in the respiratory tract. Total particulate tar dose is approximately 0.2% that of mainstream smokers, compared with relative cotinine levels of approximately 1% (2), and an

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increased relative cancer risk of 10-30% reported for 'passive' smokers relative to non-smokers (1). It would seem, therefore, that the increased lung cancer risk from exposure to ETS as estimated from epidemiology cannot be explained by dosimetric estimates of tar deposition. However, a study of lung cancer in Swedish women, recently published by Svensson *et al* (14), shows a higher proportion of adenocarcinoma cases relative to small cell and squamous cell carcinoma in never-smoking women compared with smokers, where the pattern is reversed. It is therefore interesting to note that tar particulate deposition occurs in the same pattern, with the majority in the TB region in smokers, and the majority in the P region in 'passive' smokers. Finally, it has been suggested (15) that other suspected but unconfirmed smoking-related health risks such as increased incidence of cardio-vascular disease or cancers other than respiratory tract may be linked to levels of systemic uptake of smoke constituents in ETS. This should also be considered in the light of results reported here, where the majority of ETS by mass is present in the vapour phase and deposits very efficiently, with more rapid uptake than when associated with smoke particles in mainstream smoke. Naturally, our results apply only to the specific case of iodoheptadecane uptake and the efficiency of deposition of other vapour phase compounds will be determined individually, with respect to solubility and other chemical behaviour (16). However, the general principle of more efficient and more rapid deposition of vapours is still likely to apply for most compounds.

CONCLUSIONS & RECOMMENDATIONS

In conclusion, we have shown that cigarette smoke, both mainstream and sidestream, is a dynamic aerosol and that its chemical and physical properties must be taken into account when considering dosimetry of tar in the respiratory tract, particularly with reference to relative risk estimates. This is especially important when considering that 70% of ETS by mass is in the vapour phase. Further work is required on risk estimates of individual compounds within tar with respect to their individual physical and chemical properties.

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**COMMENTS OF R. J. REYNOLDS TOBACCO COMPANY
ON HEALTH EFFECTS OF PASSIVE SMOKING - ASSESSMENT OF
LUNG CANCER IN ADULTS AND
RESPIRATORY DISORDERS IN CHILDREN
(EPA/600/6-90/0064 - External Review Draft)**

October 1, 1990

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RJR APPENDIX B

**COMMENTS OF THE R. J. REYNOLDS
TOBACCO COMPANY ON APPENDIX C
TO THE HEALTH ASSESSMENT -
DOSIMETRY OF ENVIRONMENTAL TOBACCO SMOKE**

September 1990
R. J. Reynolds Tobacco Company
Comments - RJR Appendix B

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COMMENTS ON EPA'S APPENDIX C

Appendix C of the Health Assessment is concerned with the estimation of organ dose due to exposure to chemicals present in tobacco smoke. A mathematical model is proposed which the EPA claims is applicable to both active smoking and ETS exposure. The dosimetry models discussed in Appendix C are simplistic, improperly applied and contain many errors. At the very least, Appendix C should be rewritten to include a discussion of the consequences of simplifying assumptions, errors should be corrected, and a more realistic sample calculation should be supplied.

According to the executive summary of the EPA Report to Congress on Indoor Air Quality [1], the EPA Health Assessment document was written to support EPA efforts to "provide the public with an understanding of the hazards of ETS as well as reliable methods for risk mitigation." Considerable controversy surrounds the potential health effects of ETS exposure. Due to the influence of public opinion, ETS issues have become as much or more political in nature than they are scientific. The responsibility of the EPA is to present an unbiased scientific perspective on the issues surrounding ETS exposure without being swayed by politics or public opinion. The EPA's Health Assessment should provide a complete review, giving equal consideration to the strengths and weaknesses of data which support both sides of the issue.

Unfortunately, the discussion of exposure assessment presented in Appendix C of the Health Assessment suffers from improper assumptions and does not comprise an objective

literature review. Both faulty assumptions and improper calculations characterize the biologically active dose calculations presented in Appendix C. Worse yet, equations derived for the purpose of estimating biologically active dose were ignored or misused. Lung intake is proposed as the best available estimate of exposure - despite the severe deficit of information about ETS component concentrations. Furthermore, exposure estimates are *assumed* rather than *measured*. (Frequently, measurements do not exist because concentrations fall below analytical detection limits.) Exposure estimates used in the calculations are biased high and disagree with published data. In addition, correlation of sidestream component concentrations with ETS concentrations were not performed using representative real-world concentrations. The calculations of exposure are biased and serve only to add "shock value" to this EPA draft document. Calculated concentrations poorly represent the real-world ETS exposures reported in the literature.

Comments on C.1

On pages C-1 and C-2 of the document the EPA discusses potential etiological agents found in mainstream smoke. While interesting, this information is irrelevant to a discussion of ETS risk. Mainstream concentrations of these substances are many orders of magnitude greater than those experienced by exposed nonsmokers. No substantiating animal bioassays have been performed at characteristic ETS concentrations for the compounds listed on page C-2 to determine whether they are causative agents for cancer of the lung. In the absence of realistic (ETS-level) bioassays, one cannot conclude that the compounds listed are etiological agents in ETS. Furthermore, in many environments ETS is not a major source of many compounds listed on page C-2 [2-10].

Comments on C.2

The dosimetry models discussed in Section C.2 suffer three deficiencies. First, the models are simplistic. Physical, biochemical and biological phenomena are treated in an empirical fashion with little attention given to underlying physico-chemical complexity. Empirical models are not *de facto* objectionable, but their application to real systems must be accompanied by a full explanation of model limitations and careful evaluation of error propagation. Accurate input parameters are of paramount importance in empirical models. Second, the model development section contains mis-statements and unjustified approximations. Some equations contain typographical errors making them difficult to calculate or analyze fully. Finally, calculations performed to illustrate the model's utility are impaired not only by drastic simplifications, but also by highly questionable parameter estimates. Each of these shortcomings is addressed in more detail below.

Deficiencies in the Empirical Model:

The process by which the human lung (and subsequently, other organs) is exposed to a biologically active dose of environmental aerosol involves a coupling of complex physical and chemical mechanisms. [See, for example, contributions in reference 11.] These include the fluid dynamics of aerosol flow from the environment into the lung passageways; component migration through the circulatory system; microscopic aerosol dynamics of particle/vapor evolution; mass transport by diffusion, convection, sedimentation, inertial impaction, etc.; thermodynamics of chemical reaction and heat transfer; biophysics of tissue/cellular transport; and biochemistry of chemical-receptor binding. This excludes the preliminary but equally

important factors which control aerosol dynamics in the environment, *e.g.*, ventilation, source-sink location, filtration, etc. The discussion of exposure-dosimetry modeling in Appendix C document gives little or no attention to any of these influences.

Rather than approach the exposure-dose phenomenon from a "first principles" perspective, the EPA Risk Assessment has chosen an empirical description. Empirical models are often necessary substitutes for systems whose complexity precludes a more comprehensive treatment. Human exposure-dosimetry phenomena fall within this category. However, when an empirical approach is selected, a cautionary rule applies: The more empirical the model, the more limited is the application. For example, reducing a dynamically evolving ETS particle size distribution to a single average-diameter, uniformly dispersed, constant-concentration aerosol severely limits the environments for which the model is appropriate [page C-17]. If cancellation of effects or time-averaging serves to increase confidence in the approximation, then justification should be provided. Simplification for the sake of mathematical tractability alone is never acceptable. At best, the EPA provides scant support for the simplifications invoked. The model is simplified to an even greater extent when sample calculations are presented.

Empirical models usually require input of experimentally measured parameters, *e.g.*, initial concentrations, transfer coefficients, partition coefficients, etc., in addition to more fundamental physical and chemical constants, *e.g.*, diffusion coefficients, molecular weights, vapor pressures, etc. For this reason, the accuracy of input data and propagation of error from input assumptions are very important. The EPA does not provide propagation-of-error analysis, however simple, for any equations. This is especially troubling since many of the models' input parameters are of questionable accuracy or even validity.

Mis-statements, Approximations and Errors:

The development of the mathematical exposure-dosimetry model in pages C-4 through C-17 of the document contains mis-statements, unsubstantiated approximations and simple errors. Several are enumerated below.

- (1) Concentration is assumed, beginning in equation (1), to be a function of time only. Later it is assumed to be constant. For particulate matter, this is a gross approximation both within the lung and in the external environment. [See, for example, reference 12.] Properly, concentration is a function of both time and position; i.e., $C(r,t)$. This approximation should be clearly stated in the text.
- (2) The upper integration bound on the inner integral in equation (14) should be "t" rather than "T."
- (3) Following equation (15) in the text, the EPA states, "When K is unknown (as is true for ETS), it is ignored and the dose is replaced by the integral of the organ burden, IB." This is a misleading statement. In fact, the EPA has assumed a specific value of "K"; i.e., "1." A critical factor cannot be "ignored" or arbitrarily equated to a convenient value because the true value is unknown.
- (4) In the discussion of "biologically active dose" on page C-11, the EPA states, "In general, k_A will be the fraction of the inhaled chemical biotransformed into the active form." More correctly this should read, "In the simplest approximation, k_A will be"
- (5) On page C-11 the EPA states, "For most chemicals (particularly those in ETS), k_A is unknown and B_B or D_B must be approximated by B or D as described

earlier." This statement is misleading for two reasons.

- i) It contradicts the EPA's previous statement on page C-4: "Since the incidence of effect per unit concentration can be quite different for [gas-phase and particle-bound nicotine], total exposure intensity may act as a poor measure of risk."
 - ii) The EPA has argued that " $D_B = k_A D$ ". This conclusion that D_B must be approximated by D when k_A is unknown, implies the choice of a specific value for the unknown; i.e., $k_A = 1$. By the EPA's own interpretation of k_A , this is equivalent to the assumption that *all the inhaled chemical* is transformed into a biologically active form.
- (6) Equation (16) likely represents an oversimplification of the actual processes taking place in the lung. As stated in the discussion on page C-10, retention may be approximated either by a single exponential function or by a sum of exponential functions. A simple, realistic scenario suggests that a sum of exponential functions is more appropriate. Suppose the biologically active constituent is actually a metabolite of the inspired substance. In that case, the retention function is a combination of formation rate of the compound, transfer rate of the parent compound from the organ to the blood, and elimination rate of the biologically active compound. This scenario is probably characteristic of many compounds in ETS alleged to have detrimental health effects. For example, polycyclic aromatic hydrocarbons (PAH) and nitrosamines exhibit no inherent biological

activity [13-16]. Rather, it is their metabolites that are associated with biological effect. Depending upon the complexity of metabolite kinetics, the assumption of a single, exponential retention function could introduce significant error. The use of simple exponential functions also assumes, without substantiation, that metabolism rate and transport across cell membranes are governed by first-order mechanisms.

- (7) Equation (17) should read " $B(T) = \dots$ " rather than " $D(T) = \dots$ ".
- (8) Nicotine and cotinine are mentioned extensively throughout Appendix C, although neither has been associated with any adverse health effects at concentrations present in ETS. Furthermore, the discussion adds nothing to the understanding of the biological activity of ETS. Many studies suggest that nicotine is of little value in predicting exposure to other ETS constituents [17-22]. The excessive attention paid to nicotine gives the reader the impression that nicotine is a typical ETS component; an impression which is not substantiated in the literature. In fact, the EPA states on page C-15, "If the dose to the blood is calculated for nicotine, therefore, the dose to other organs or tissues may be obtained by multiplying the ratios in Table C-1. It is unlikely, however, that the same ratios will apply to other chemicals in ETS." A more complete discussion of nicotine and cotinine as biomarkers is presented in Section I.F. of the main RJR comments.
- (9) In Figure C-2 on page C-13, the deposition fraction labels $f_{TB,GI}$ and $f_{NP,GI}$ are missing from the appropriate "arrows."

The summary on pages C-15 to C-17 is also misleading. The EPA claims that measurement of exposure to "given *chemical[s]* in ETS can take several forms." This is a peculiar statement as most of the 4700 compounds alleged by the EPA to be in ETS [23] have never been measured in ETS. In addition, most of the "43 known or suspected carcinogens identified in tobacco smoke" [The Health Assessment at 2-1] have not been detected in real-world ETS. Even if detected, the question of origin remains unanswered. What fraction of the substance originated in ETS, and what fraction came from another source? Some examples of alternate sources for alleged carcinogens in indoor air are given in references 2-5,8,9.

The calculation of exposure-dose outlined in the summary is simplistic. For those compounds which can be measured, exposure intensity, cumulative exposure and lung intake can be estimated under certain conditions. For example, external ETS aerosol dynamics must be simple enough to permit measurement of environmental concentrations, and volumetric breathing rate must be reasonably constant over the exposure period. But for most compounds, total lung uptake "U", or regional uptakes, cannot be calculated because the fraction of material deposited in the lung, "f", is unknown. Neither total lung burden "B", nor regional burdens, can be calculated because the retention function "R" is unknown. Although the EPA points out that "R" may be *estimated* for particles, no references for its use with other ETS components are given. Subsequent calculation of integral organ burden "IB" is similarly flawed.

Items 7 through 12 in the summary deal with dose to the lung, dose to other organs, and biologically active dose. The gross assumptions and myriad unknowns which characterize these "measures" make useful calculations impossible. In light of the absence of critical information, the EPA concludes on page C-17, that "intake" is by default "the best available measure of

exposure." But the extent of their own derivations suggests that intake is a relatively poor measure of exposure or risk. In addition, alternate routes of intake may provide dose contributions for compounds present in ETS. For example PAHs and nitrosamines may originate in foods rather than air [6,24-26]. All sources of exposure must be carefully considered when relative risk assessments are made.

Finally, the EPA states, "The measure of exposure to a chemical depends upon the level of available information." This statement and those following read like an apology for failing to attempt a more rigorous mathematical description of exposure and dose. It is not the "measure of exposure" that depends upon the "level of available information," but the *accuracy of the approximation*. What the EPA refers to as an "upstream" measure is nothing more than a less accurate approximation of an equation which is already empirical.

Comments on C.3

Section C.3 purports to estimate intakes based upon assumed exposure conditions. But several erroneous assumptions tend to invalidate the results. Errors include miscalculation of exposure conditions, use of inapplicable ratios, and biased exposure data which have not been corrected for non-ETS, *i.e.*, background, sources of compounds. Several specific problems are identified below.

The unnumbered equation on page C-18 is approximate, even within the context of the given empirical description, and depends upon all the prior approximations which went into the derivation of the equations in Section C-2. It is mathematically exact only when the burden function, $B(t)$, is constant; an unlikely prospect. If $R(t)$ is of exponential form, as the EPA

argues in the derivation of equation (18), then the correct dose ratio is given by

$$\frac{D^*}{D} = \frac{C^*}{C} \cdot \frac{[T - (1 - e^{-\lambda T})]}{[T - (1 - e^{-\lambda T})]}$$

The equation presented on page C-18 is acceptable only for small values of the exponential argument " λT ." The EPA should clearly state the approximate nature of this equation.

According to Appendix C, a constant ETS exposure of $200 \mu\text{g}/\text{m}^3$ of respirable particles was calculated from tables in the NRC report [27]. Figure 1 shows the predicted concentration vs. time profile for particles assuming 26 mg RSP per cigarette, 150 m^3 room volume, air exchange rate of "one per hour",

and twenty cigarettes smoked over a 24-hour period (all conditions as assumed in the document). The calculation also assumes a first-order decay of particles [17,18], and a 9 minute per cigarette smoking duration. Figure 1 shows a 24-hour period following the preliminary smoking of three cigarettes under the stated conditions (so that steady state

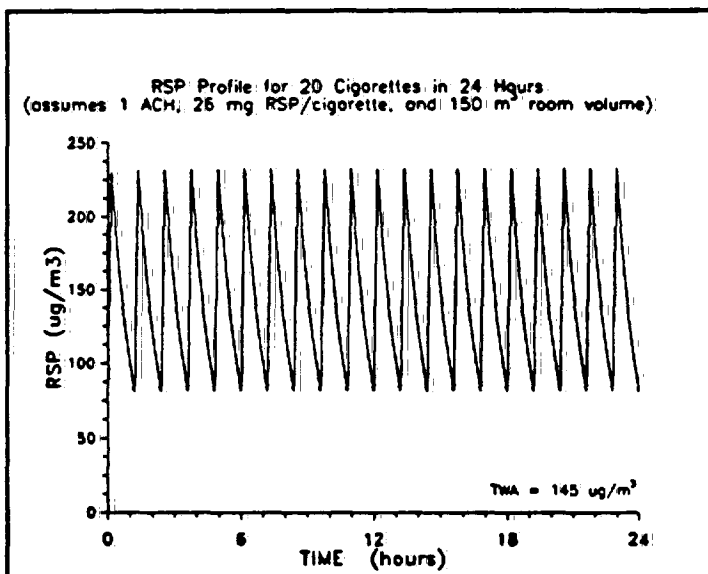


Figure 1: Modeled RSP vs time profile for smoking 20 cigarettes in 24 hours. Room volume, RSP per cigarette and air exchange rate from EPA appendix C. Nine minutes smoking time per cigarette was assumed.

maximum and minimum concentrations are reached). It is clear from these results that the assumption of a constant RSP concentration cannot reasonably be inferred from the smoking

pattern described in Appendix C [page C-18]. The RSP concentrations in Figure 1 vary between $81 \mu\text{g}/\text{m}^3$ just before smoking to $232 \mu\text{g}/\text{m}^3$ immediately after smoking. The EPA-estimated concentration of $200 \mu\text{g}/\text{m}^3$ is 38% higher than the average value of $145 \mu\text{g}/\text{m}^3$ obtained from this model. It should be noted that smoking duration is a sensitive variable in the calculations. For example, a smoking duration of 5 minutes yields an average concentration of $156 \mu\text{g}/\text{m}^3$ (higher by $\sim 8\%$, but still much lower than the value quoted in Appendix C). The concentrations of sidestream particles used in the EPA calculations are higher than those reported by other researchers [28]. Also, the cigarette tested is not identified. If it is a non-filter cigarette, it poorly represents the average cigarette smoked in the United States. Adams *et al.* have shown that non-filter cigarettes produce considerably more sidestream particles than a perforated filter cigarette smoked under the same conditions. Furthermore, the sidestream particle concentration in the EPA document is 7 to 83% higher than the values reported by Adams [28].

Temporal concentration variations, similar to those depicted in Figure 1, are corroborated by experimental measurement. Eudy, *et al.* have conducted field studies in which concentrations of ETS components, such as nicotine, vary widely with time [29]. Test chamber studies have also yielded data which argue against the use of constant concentrations in exposure calculations [30,31]. The EPA has ignored this factor and the pertinent references.

In an earlier publication, the EPA has stated that "ETS diffuses rapidly throughout buildings and homes" [32]. (The EPA has mis-used the word "diffuse" in this context. It is likely that they intend the broader term "disperse.") If this is the case, then a volume of 150 m^3 may be too small. An average home volume of 353 m^3 [33] has been measured elsewhere for

indoor air particulate studies. Correcting for this value reduces the exposure estimate by approximately 50%. Finally, the "per-minute" ventilation rate used in the calculations is considerably greater than averages published by ASHRAE [34]. The EPA's choice again leads to overestimation of exposure, uptake, etc.

Table C-2 presents SS/MS mass ratios of substances found in sidestream and mainstream "tar." Equating sidestream "tar" to ETS "respirable particles" is incorrect. Previous EPA research has shown an ETS particulate yield of 10 mg per cigarette [35]. This is less than half the 26 mg value quoted in Appendix C for sidestream "tar." Computing nonsmoker exposure to RSP on the basis of sidestream "tar" then results in overestimation. In addition, little is known about how "tar" differs chemically from respirable particles. The "tar" ratios presented in Table C-2 are probably not applicable to ETS respirable particles. Furthermore, quoting *average* data from tables in the NRC report [27] can mislead the reader. In fact there is a large spread of ratios in the NRC report which leads to significant error propagation in the EPA's exposure calculations.

As a result of wide differences in sidestream concentrations (derived from different testing methods), and wide differences among sidestream emissions (as a function of cigarette type) [28], the ratios presented in Table C-2 of Appendix C have little or no meaning when applied to real-world environments. In addition, this table either incorrectly transcribes entries from the original source [27] or miscalculates "averages." Specifically, the SS/MS ratios of three compounds are incorrect: 3-methylpyridine, 3-vinylpyridine and hydrogen cyanide. Following the EPA's technique of averaging the high and low values of a reported range (in itself a questionable practice) to achieve a "representative value," the proper ratios for these substances should be 8,

30 and 0.18, respectively (as opposed to the reported 13, 10 and 30). The ratio reported for hydrogen cyanide is two orders of magnitude greater than the properly calculated value.

As argued above, the concentration factor "200 μ g" in equation (25) exaggerates nonsmoker exposure, and the results presented in Table C-3 are consequently biased. Confidence limits associated with each entry are large and should be included in the table. In addition, equation (25) tacitly assumes both that ETS chemical component concentrations track RSP concentrations, and that the sidestream aging process is unimportant. Neither of these assumptions is valid [see, e.g., 17,36-39].

The data in Table C-4 are not representative of the majority of current indoor air quality research. Several reviews and original papers give widely different values, especially for measurements taken under more typical smoking conditions [e.g., references 40,41]. On page C-21, a comparison of ratios obtained from Tables C-3 and C-4 is presented. Several important considerations are ignored. As discussed in preceding paragraphs, the numbers presented in Table C-3 were obtained from data averages covering a wide range of smoking conditions. More important, the numbers presented in Table C-4 are concentrations measured in indoor air. Although ETS may be a source of many of the compounds in the tables, it does not necessarily contribute a major portion to quantities measured. The numbers presented in the table, and used in calculating ratios, are *total* benzene, PAH, n-DMA, etc. No attempt has been made to apportion the amount of each substance into ETS and non-ETS sources. Attributing all substances measured to a common ETS source greatly misrepresents the complex mix of compounds present in indoor air. The Appendix states that "the reason for the increase in benzene after aging is unknown." The most likely reason is that in the environment sampled,

ETS contributed only minor amounts of benzene; non-ETS benzene was improperly attributed to ETS.

A third severe problem with arguments presented on page C-21, is the "normalization" of all ratios to nicotine. ETS nicotine is a poor predictive marker for other compounds in ETS [18-20,42]. Recent investigations have shown that this problem arises in part from its ready tendency to adsorb on and desorb from surfaces [18,22]. Ratios of nicotine to other ETS components vary widely, *e.g.*, 4.4 to 53 $\mu\text{g RSP}/\mu\text{g nicotine}$ [20,43]. The use of nicotine for standardizing ratios of other ETS constituents leads to overestimation of exposure by as much as 1000% in some instances.

The statements on page C-26 are irrelevant. Doses of nicotine calculated for exposure to ETS are trivial. Although nicotine may be a precursor of NNN and NNK in tobacco leaf [44-46] and it has been speculated by Hoffmann and Hecht (1989) to be a precursor of NNK *in vivo*, there is no evidence to support this speculation. In fact, there is evidence against *in vivo* conversion, and chemical studies show that the nitrosation reaction is so slow as to make such *in vivo* conversion highly unlikely [47, 58]. The statements regarding NNN and NNK are likely to mislead the reader into mistakenly believing that nicotine taken into a nonsmoker's body will form these compounds. Finally, nicotine is problematic as a quantitative ETS biomarker. Some problems have been discussed previously in other sections of this review. See Section I.F. of the main RJR comments.

On page C-28, the EPA estimates the uptake of RSP by a nonsmoker. Alternative calculations are presented in RJR's Sample Calculation in this review. RJR's sample calculations are based upon a time budget for a nonsmoker who works in a smoking-allowed office and who

is married to a smoker. These assumptions will overestimate ETS exposure by the population at large. Concentrations used are obtained from studies involving hundreds of measurements taken in real-world settings. Based on RJR's Sample Calculation, the nonsmoker so described has an uptake of $\sim 269 \mu\text{g}$ RSP per week ($38 \mu\text{g/day}$). This amounts to $\sim 0.02\%$ of the RSP exposure of a smoker during the same period of time. These results illustrate not only shortcomings of the EPA calculations, including input parameter bias, but also the variations produced by other estimations.

Comments on C.5

The calculations in Section C.5 follow from the biased calculations in Section C.3. At the very least, Section C.5 should be recalculated taking into account comments provided in this review. Use of the same half-life for particle removal from the P region of smokers and individuals exposed to ETS is inaccurate. On page C-30, the flow of mucus, and hence the removal of particles, in the TB region of smokers is reduced by a factor of 2. It does not seem likely that the removal of particles from the P region of nonsmokers' lungs would be equivalent to the removal from smokers' lungs. Use of an alveolar half-life in smokers to represent nonsmokers may lead to an overestimation of internal organ burden in nonsmokers.

Data relating to nicotine on page C-30 are irrelevant. Nicotine is a hygroscopic compound and is miscible with water [48]. The absence of hygroscopic growth of ETS particles described on page C-28 suggests that ETS particulate matter, unlike mainstream particles, is composed primarily of hydrophobic compounds. For this reason, equating the behavior of nicotine to ETS particles in the lung is inappropriate.

The calculation of daily internal organ burden for RSP on page C-31 is flawed for the reasons described throughout this review. Arguments presented earlier suggest that the propagated error at this point is large for the values presented in the tables. Absence of reference to original manuscripts, failure to substantiate the validity of assumptions, and biased review of exposure data used in the calculations are disturbing. This section should be discarded. Minimally, it should be rewritten with attention paid to verification of assumptions and propagation of error.

Comments on C.6

See Section I.F of the main RJR comments.

RJR SAMPLE CALCULATION

Calculation of weekly ETS-RSP exposure of a male nonsmoker who works in an office in which smoking is permitted and takes place and who is married to a smoker.

Given:

1 week = 10,080 minutes

Assume 90% of time is spent indoors

∴ 9072 minutes spent indoors

8 hours sleep per day = 3360 minutes

9 hours per day at office = 2700 minutes
(8 hr. workday + 1 hr. for lunch)

1 hour per day commute = 300 minutes

5 hours per week in restaurant = 300 minutes

Remainder of time awake at home = 2412 minutes

Weekly Exposure Estimate:

Sleeping:

$$7.5 \frac{\text{t}}{\text{min}} \times 3360 \frac{\text{min}}{\text{week}} \times 10^{-3} \frac{\text{m}^3}{\text{t}} \times 33 \frac{\mu\text{g}}{\text{m}^3} = 831 \frac{\mu\text{g}}{\text{week}}$$

At Work:

$$9 \frac{\text{t}}{\text{min}} \times 2700 \frac{\text{min}}{\text{week}} \times 10^{-3} \frac{\text{m}^3}{\text{t}} \times 41 \frac{\mu\text{g}}{\text{m}^3} = 999 \frac{\mu\text{g}}{\text{week}}$$

In Restaurant:

$$8 \frac{l}{min} \times 300 \frac{min}{week} \times 10^{-3} \frac{m^3}{l} \times 59 \frac{\mu g}{m^3} = 142 \frac{\mu g}{week}$$

Remainder at Home:

$$9 \frac{l}{min} \times 2412 \frac{min}{week} \times 10^{-3} \frac{m^3}{l} \times 33 \frac{\mu g}{m^3} = 716 \frac{\mu g}{week}$$

Commute:

No Exposure

Total Exposure:

2,685 μg RSP/week from ETS.

Assuming a 10% retention [49]:

269 μg ETS RSP/week retained.

Total dose to smoker from EPA document:

1,344,000 μg RSP/week.

Relative dose to exposed nonsmoker:

A nonsmoker's tobacco smoke particle uptake is only 0.02% of smoker's uptake.

Note:

Restaurant and office data are based upon arithmetic mean of data obtained from previous surveys. The data are lognormally distributed, therefore the arithmetic mean will overestimate the exposure to the average person. Use of a geometric mean is a more appropriate method for estimating average exposure. When the geometric mean is substituted into the previous equations, the nonsmoker relative dose is reduced still further.

Sources:

ETS Exposure in the home was taken from the data of Leaderer (survey of 393 homes) [33].

ETS restaurant data are from a series of surveys of 239 restaurants in the US and Ottawa [50-55] for which an apportionment of RSP to ETS and non-ETS fraction was available [56].

ETS office data are from a series of surveys of 206 offices in the US and Ottawa [50,52,55,57]. Smoking was permitted and observed in each office included in the surveys. RSP apportionment [56] information was available for each office used in the calculations.

Breathing rates were obtained from the ASHRAE Handbook, Fundamentals Volume [34].

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RJR APPENDIX C

**COMMENTS OF THE R. J. REYNOLDS
TOBACCO COMPANY ON APPENDIX D
TO THE HEALTH ASSESSMENT -
ALTERNATIVE APPROACHES FOR ESTIMATING
THE YEARLY NUMBER OF LUNG CANCER DEATHS
IN NONSMOKERS DUE TO ETS BASED ON
DOSE RESPONSE MODELING**

September 1990
R.J. Reynolds Tobacco Company
Comments - RJR Appendix C

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COMMENTS ON EPA APPENDIX D

Appendix D of the Health Assessment discusses potential methods for the derivation of a dose-response model for ETS exposure and age-specific lung cancer death rates. Appendix D presupposes that a causal connection has been established between lung cancer and ETS exposure. No such causal connection has been established, the appendix should be deleted from the Risk Assessment. However, since the EPA solicits comments, we have reviewed this appendix and provide comments on several shortcomings.

The Agency identifies three elements that are required to generate dose-response modeling approaches to directly estimate the "number of lung cancer deaths in nonsmokers attributable to ETS": (1) the distribution of the time-weighted exposure of ETS in a nonsmoking population, (2) the age distribution of the nonsmoking population, and (3) a mathematical dose-response model describing the relationship between the age-specific lung cancer rate and the independent variable age, sex, race and ETS exposure. The Health Assessment at D-1. The Agency suggests that it has already collected sufficient information with regard to elements one and two to perform the modeling. Nowhere is that information presented. The assertion that the time-weighted exposure of ETS in the nonsmoking population is known with certainty is baseless. Even less is known about time-weighted ETS exposure to specific age groups. Measurement of ETS is a complex undertaking for which no consensus methodology exists.

Appendix D is devoted to suggesting alternative approaches for fulfilling the third element described above -- developing mathematical dose-response models. The three proposed general approaches for deriving ETS dose-response models are:

1. Establish a dose-equivalent relationship between ETS and a positive control such as inhaled benzo[a]pyrene (B[a]P) which has an animal-based inhalation cancer dose-response model associated with it. Heavy use would be made of animal carcinogen test results in this approach. This approach will be subsequently referred to as the Relative Potency Approach ("RPA").
2. Establish an equivalency relationship between the number of cigarettes smoked per day and ETS exposure levels in mg/m³ inhaled air. This relationship would then be used to estimate risk based on direct state-of-the-art cigarette smoking dose-response model obtained from multiple sources of epidemiological data. This will be referred to as the Cigarette-equivalent Approach ("CEA").
3. Use ETS epidemiological studies where a dose-dependent increase in the risk of nonsmoking women is associated with ETS. This will be referred to as the Direct Approach ("DA").

The Health Assessment at D-2. The CEA presumes: that smoking causes lung cancer, that the epidemiologic data are quantitatively accurate and not confounded, and that ETS exposure is a quantitative variant of smoking. None of these assumption is accurate. Decades of research have failed to prove that smoking causes cancer and, as demonstrated by RJR's comments in Section II. G., ETS exposure cannot be quantitatively related to cigarette smoking. As the Agency concedes, there are no data available to derive an ETS dose-response model using the DA. In fact, the absence of those data is a fundamental flaw in the Agency's Health Assessment. RJR's further comments are restricted to the RPA.

THE RELATIVE POTENCY APPROACH

The Agency attempts to describe relative carcinogenic potency of ETS compared to B[a]P by using the data collected by Grimmer *et al.*, 1988, in a rat implant study. The Agency then suggests a number of alternatives upon which a standard inhalation dose-response model could

be based: (1) hamster inhalation B[a]P dose-response, (2) rat inhalation diesel exhaust dose-response, and (3) human inhalation coke-oven response.

B[a]P

The Agency relies on the work by Grimmer *et al.*, 1988, to derive the relative carcinogenic potency of ETS to B[a]P. This reliance is misplaced. That study employed an animal model using beeswax pellets that were surgically implanted by thoracotomy into rat lungs. The method is an extreme, highly artificial approach that does not in any way simulate the qualitative or quantitative aspects of smoking or ETS exposure, and human target doses cannot be predicted from this model. The beeswax pellet itself provides a strong foreign-body tissue response in rat lungs, and this would be expected to contribute to tumor development. The pellets are not passive delivery devices. In earlier work (Hirano *et al.*, 1974), two control animals with beeswax pellets alone developed squamous metaplasia, which indicates the degree of irritation produced. Therefore, data obtained using the beeswax technique are totally inappropriate for quantitative risk assessment.

The EPA ignores other work on beeswax implantation (Grimmer *et al.*, 1987a) that should be included as part of Table D-1. This work has shown that the contribution of B[a]P to total carcinogenicity of flue gas condensate is less than 2%. Data are also available on diesel exhaust condensate (Grimmer *et al.*, 1987b).

The lung implant technique used by Grimmer does not allow accurate estimates to be made of the actual dose. In addition, as the EPA points out, the exposure levels will most likely exponentially decrease with time. Multiple exposures cannot be used. Overall, the technique is very non-representative of any human exposure. The Office for Science and Technology

Policy (1985), in its review of carcinogenesis, points out that the data derived from such highly experimental systems are not relevant to human exposure. The OSTP uses as examples pellet implants in urinary bladders and subcutaneous injections in experimental animals, which are analogous to the lung implant studies.

The Agency suggests at D-6 that ETS condensate was implanted in the rats' lungs. In fact, sidestream smoke condensate was used. EPA's Appendix D presents many details of the work that are not in fact present in the original Grimmer article, *e.g.*, duration of study, method of introducing the implant, and the histological typing of the neoplasms. The Agency should describe the source of this unpublished information. In addition, the EPA specifically neglects to mention one of the data sets from the original Grimmer paper, *i.e.*, lack of a strong dose-relationship.

Tables D-2 and D-3 import historical lung tumor control data from a different laboratory (Goodman, 1980). The combined data set is used in the establishment of a model. This combination of data from different laboratories in different countries in different years using different pathologists is scientifically unacceptable.

The conclusion reached by the EPA, that the implant technique can be used to estimate the relative potencies of other complex PAH mixtures including, by implication, ETS, is invalid.

Hamster Inhalation B[a]P Dose-Response

Only a single group of workers (Thyssen *et al.*, 1981) have shown inhaled B[a]P to cause cancer. Several other groups have failed to replicate the observations (Pepelko, 1984). Anatomically, the cancers reported by Thyssen *et al.* to occur as a result of B[a]P inhalation were

not lung cancers. They were neoplasms of the nasal cavity, larynx/pharynx, trachea, esophagus, and forestomach and so are irrelevant to any discussion of lung cancer.

No evidence is given on the histologic typing of the neoplasms in the Thyssen B[a]P inhalation experiment; they could have been non-malignant. The EPA statement that careful histopathological examinations were made on each animal is not supported by the description or the published report.

The data in Table D-5 (the number of hamsters with one or more malignant laryngeal or pharyngeal tumors) cannot be obtained from the Thyssen paper to which the table refers. Other results presented in the table including lifetime exposure, average survival time, animals examined, are different than those published in Thyssen's paper. Moreover, the Thyssen paper is deficient in many technical areas and should be viewed as a technical note providing uncorroborated data -- not as a reference method for use in extensive modelling. The modelling technique used and quoted by the EPA has not been published and comments can therefore not be made.

The value of models based on inhaled B[a]P is thus questionable. The conclusion reached by the EPA on the use of Relative Potency Approach (RPA), namely that such an approach can be used to generate comparative data on ETS, is not valid for the reasons given above.

Diesel Exhaust Study

EPA's Appendix D reviews only cursorily the diesel exhaust inhalation study of Mauderly (1987). This limited review, only 21 lines of text, is despite the fact that the diesel exhaust study was designed to be a pulmonary carcinogenicity study, and uses a material which in some respects is more similar to ETS than the other described approaches (the EPA document uses the

ambiguous phrase "the PAH-matrix is reasonably similar to the type one might expect with human exposures"). In exposures of up to 30 months (7 h/d, 5 d/w) at RSP concentrations at least 100-fold higher than typical ETS concentrations, i.e., 35 mcg/m³, the diesel study showed no increase in malignant lung tumors. Only in the high exposure groups (200 times typical ETS RSP concentrations) were malignant tumors found. The diesel exhaust data show that the rat can respond positively in an inhalation carcinogenicity study, the test material producing a few malignant tumors. However, such results are not found at lower concentrations, lower but still much higher than typical ETS concentrations. At doses of diesel exhaust, 100-fold greater than ETS exposure, no differences were noted from sham exposed animals. The minimal attention to a key paper, and a lack of an examination of the dose response and a comparison with likely ETS exposures, is a misrepresentation of the relevant facts by the Agency.

Conclusion

The models presented rest on unfounded assumptions and scientific data which, even if they can withstand peer scrutiny, are irrelevant to human ETS exposure. Appendix D does nothing to inform the public about potential health-related effects of ETS. Instead it is an arbitrary exercise which is an abuse of the Agency's discretion because its existence is an attachment to the Health Assessment implies that it contains useful information on that subject. It should be withdrawn.

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EXCRETION OF MUTAGENS IN HUMAN URINE AFTER PASSIVE SMOKING

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(Received 6 January 1983)

(Accepted 17 January 1983)

SUMMARY

Eight non-smokers were experimentally exposed to cigarette smoke by staying in a poorly ventilated room together with heavy smokers for 6 h. Air samples were taken and the extract appeared to contain mutagenic substances. This is in accordance with the presence of carcinogens in tobacco smoke. ~~Inhalation of the contaminated air by the passive smokers resulted in an increase in the urinary excretion of products mutagenic in the *Salmonella* microsome assay.~~ This observation suggests that there is a causality in the association between increased cancer risk and passive-smoking, as was found by other investigators.

INTRODUCTION

It is a question of far-reaching significance, whether passive inhalation of tobacco smoke has only annoying, reversible effects like irritation of the eyes and the respiratory tract, or can cause permanent injury to health. Does passive smoking on the long run lead for example to an increased risk of cancer?

In 1981 Trichopoulos et al. [13] reported results from a case-control study demonstrating a statistically significant association between husbands smoking and women's cancer risk. About the same time, Hirayama [8] came to the same conclusion on the basis of a more extensive epidemiological investigation. He stated that passive and involuntary exposure to cigarette smoke markedly increases the risk of lung cancer. Contrary to these reports Garfinkel [7] failed to show such a statistically significant relationship.

Recently, the discrepancies between the results of these epidemiological studies have been discussed at length [10,11]. In particular, objections were

made to the quantitative interpretations with respect to the increased risk of lung cancer in passively smoking women.

In the present investigation an attempt was made to study the causality of an increased cancer risk due to passive smoking. Thereto, urine samples of passive smokers staying in a smoky room for 6 h were screened for the presence of mutagens. Measurement of urinary mutagenicity has been used successfully to establish environmental exposure to mutagenic or carcinogenic agents. In previous studies it was shown that active cigarette-smoking leads to the appearance of mutagens in the urine [4,17]. Other investigations are dealing with urinary mutagenicity after occupational or therapeutic exposure to carcinogenic substances [2,5,12].

MATERIALS AND METHODS

Collection of urine samples

Urine samples were collected from 8 male non-smokers and 10 smokers of either sex. The age of the subjects ranged from 25 to 35 years. It was assured that the non-smokers did not use any drugs. The non-smokers together with the heavy smokers stayed in a room (110 m³) with poor ventilation for 6 h. During this period 157 cigarettes were smoked by the smokers. Just before the exposure was started, urine was collected and discarded. Thereupon urine was collected for a 12-h period. In addition urine of non-smokers was collected for the same period the day before and the day after the exposure to cigarette smoke. After collection, the urine samples were stored at -20°C until assayed. All samples were handled at one go to avoid negative influences of variations in the assay on the final result.

Method for concentrating mutagens present in urine

Thirty percent of a 12-h urine sample was loaded on an amberlite XAD-2 column with a 4-cm³ bed volume. The column was washed 3 times with 5 ml of aqua dest. The adsorbed material was eluted with 10 ml of acetone. The eluate was evaporated to dryness under nitrogen at 60°C and the residue was dissolved in 0.45 ml of dimethylsulphoxide.

Air sampling

During the stay of persons in the room filled with smoke, a total of about 500 l of smoky air was bubbled (1.3 l/min) through 2 cylinders (in series) filled with ice-cold hexane. The same was done 1 day before and 1 day after the experiment, when no smokers were present in this room. The hexane portions were combined and evaporated to dryness, using a rotary evaporator. The final residue was taken up in 1 ml of dimethylsulphoxide.

Salmonella/microsome assay

Urine and air concentrates (0.1 ml per plate) were assayed for mutagenicity with the *Salmonella typhimurium* tester strain TA1538 in the presence of

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S9 mix [1]. Every determination was done in triplicate. After incubation of the plates for 48 h at 37°C the number of revertant colonies was counted.

S9 mix

The S9 mix was prepared according to Ames et al. [1] and contained per ml, 100 μ l of a hepatic 9000 \times g supernatant from Aroclor 1254 induced rats and a NADPH-generating system.

Statistical analysis

The experimental data (8 cases ($i = 1, 2, \dots, 8$); 3 observations x_{ij} ($j = 1, 2, 3$)) were handled as follows. In our particular case we formulated the null hypothesis: H_0 : per case there is no preference for the highest value in any of the 3 observations; and the alternative hypothesis, H_1 : per case there is a preference for the highest value in the second observation. This results in a binomial test with probability of success 0.33 if H_0 is true. "Success" is defined as "the value of the second observation being the highest".

RESULTS

Mutagenicity in urine

Urine samples of 8 non-smoking persons were collected before, during and after the passive exposure to cigarette smoke and mutagenicities were measured. The results are shown in Table 1. For 6 out of 8 persons highest

TABLE 1

MUTAGENICITY OF URINE FROM PASSIVE SMOKERS

Non-smoker	R_T/R_S -value ^a		
	Collection of urine samples ^b		
	Day before passive smoking	Day of passive smoking	Day after passive smoking
a	1.8	5.5	3.1
b	3.4	4.2	2.3
c	2.9	3.4	2.3
d	3.1	3.0	2.4
e	3.8	-2.7	2.2
f	3.1	4.2	3.2
g	2.4	3.1	1.8
h	4.0	5.1	2.9

^a R_T/R_S = Mean no. of revertants found with urine/Mean no. of revertants found with aqua dest.

^bDetailed information is given in Materials and Methods.

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values were observed at the day of passive smoking. Statistical analysis of the data revealed a significant enhancement of urinary mutagenicity during passive smoking ($P = 0.02$).

Urine of the 10 smokers was collected the day of the experiment and handled in the same way as the urine of the passive smokers. Relative urinary mutagenicity values (R_T/R_S) ranged from 9.0 to 42.0 with a median value of 23.0.

Mutagenicity in the air

During the passive smoking experiment the ambient air in the room, gradually contaminated with cigarette smoke, was tested for the presence of mutagens. A concentrate equivalent to 50 l of air, containing the cigarette smoke, revealed 155 revertant colonies per plate. Testing a concentrate of an equal volume of air from the same room the day before or the day after, when no smokers were present, revealed 12 and 16 revertant colonies per plate, respectively.

DISCUSSION

These results clearly indicate that during experimental conditions, simulating passive smoking, compounds are inhaled that lead to urinary excretion of products which are mutagenic in the *Salmonella*/microsome assay. This finding is in good agreement with the observation that the ambient air in the smoky room contained a substantial amount of mutagenic substances as compared with the background as measured the day before and the day after the experiment. The mutagenicity in the air sample is in conformity with the data about the presence of carcinogens in tobacco smoke. In 1964 Galuskinova [6] reported the presence of benzo[a]pyrene in the smoky atmosphere of social meeting rooms and restaurants. Other investigators found that the amount of some carcinogens, e.g. β -naphthylamine, in side-stream smoke is many times that in mainstream smoke [14].

Since it is generally acknowledged that most of the genotoxic carcinogens can be detected by in vitro mutagenicity tests, our results about the mutagenicity in urine of passive smokers can be considered as an indication of carcinogenic exposure. This is in support of the reports of Hirayama [8] and of Trichopoulos et al. [13], who suggested a statistically significant association between husbands smoking and women's cancer risk. Nevertheless it can be doubted whether the estimations of the relative risks due to passive smoking, as made by these authors, are realistic [11].

It is known that the incidence of lung cancer is well-correlated with the smoking habits of the people concerned [3,16]. In a previous paper we showed that there exists a relationship between the mutagenicity in urine and the number of cigarettes smoked [4]. It is an interesting question, whether urinary mutagenicity might give in some way a reflection of the relative cancer risk of smoking.

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In the urine of 8 non-smokers we found a median urinary mutagenicity value (R_T/R_S) of 3.7 during the experimental passive-smoking. The median urinary mutagenicity value of these persons on the day before and after the passive-smoking experiment was 2.8. In urine of the 10 heavy smokers (> 20 cigarettes/day) we found urinary mutagenicity values ranging from 9.0 to 4.2 with a median value of 23.0. Thus the increase in urinary mutagenicity due to the passive exposure to cigarette smoke is about 4% of the increase observed with the active smokers during the experiment. Judging from these data, it is very tempting, though speculative, to suppose that the increased risk of cancer for passive-smokers under comparable circumstances is of the same order of magnitude.

During the last decade society has become very concerned about the environmental cancer risk. Inhalation of air-pollutants, like PAHs produced in the combustion of, for instance, coal and oil, has been considered as a major cause of the increased pulmonary cancer [15]. For smokers the risk is multiplied, because the presence of many other carcinogenic substances in the cigarette smoke. It is important to note that the benzo[a]pyrene concentration of cigarette smoke is 500–1000 times higher than the average value in the city air [15]. In addition, cigarette smoke may contain inducers of microsomal oxidation by which other pre-carcinogenic substances are converted into their active forms [2,9].

The experimental results of the present study and the available epidemiological data suggest that non-smokers involuntarily inhale potential carcinogens. Strategies to control the environmental cancer problem can be only successful if the health-hazards of passive smoking are sufficiently acknowledged.

ACKNOWLEDGEMENTS

The authors are indebted to Dr. S.H.J. Veling for his assistance in the statistical analysis of the results. Financial support was obtained from the General Directorate of Labour, Dutch Ministry of Social Affairs.

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Passive smoking at work: biochemical and biological measures of exposure to environmental tobacco smoke

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Summary. Several biochemical and biological measures of tobacco smoke intake were used to evaluate exposure of restaurant personnel to environmental tobacco smoke as compared with active smokers and non-exposed non-smokers. All of the measured parameters – carboxyhaemoglobin (COHb), thiocyanate (SCN) and cotinine in plasma, cotinine and mutagenicity in urine, total white blood cell count (WBC), and sister chromatid exchange (SCE) frequency in cultured lymphocytes – were significantly elevated in the smoker group ($n = 22$) compared to the non-exposed group ($n = 20$). Work-related passive exposure ($n = 27$) was seen most clearly in the cotinine values, both from plasma (mean P-cot in passive smokers 10 ng/ml vs 5.2 ng/ml in non-exposed) and from urine (mean U-cot in passive smokers 56 ng/ml vs 8.3 ng/ml in non-exposed), but significant increases were also seen in the thiocyanate levels (mean P-SNC in passive smokers 58 μ mol/l vs 46 μ mol/l in non-exposed) and, as a preliminary finding, in total leucocyte count (in passive smokers $8.0 \times 10^9/l$ vs $6.8 \times 10^9/l$ in non-exposed). The results demonstrate that environmental tobacco smoke may be an occupational health hazard.

Key words: Tobacco smoke – Occupational exposure – Cotinine – Thiocyanate – Carboxyhaemoglobin – Genotoxicity

Introduction

Tobacco smoke contains a large number of irritative [1, 2], carcinogenic [3], and mutagenic compounds [4]. The severe health risks of smoking, mainly associated with chronic lung diseases, cardiovascular diseases and lung cancer are well

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ethylthiouracil as an internal standard [15]. Total leucocyte counts were obtained with an automated blood cell counter (Coulter). For urinary mutagenicity testing, urine samples were first standardized for creatinine to 4 mmol/l and then concentrated in XAD-2 resin for analysis of the urinary mutagenicity [16]. The mutagenic activities were analysed by the bacterial fluctuation test, using *Salmonella typhimurium* strain TA 98 with rat liver S-9-mix as the indicator system [17]. The highest volume tested, 25 μ l of the urine concentrate, corresponds to 6.25 ml of the original standardized urine sample (0.025 mmol of creatinine). The cytogenetic investigation was performed in cultured peripheral blood lymphocytes by the sister chromatid exchange technique. The cultures were set up using 0.2 ml whole blood in 4-ml cultures containing RPMI 1640 medium supplemented with 15% fetal calf serum and 15 μ M 5-bromodeoxyuridine. An acceptable amount of differentially stained metaphases for the SCE analysis, 30 to 50 per person, were obtained for 46 persons from the whole blood cultures, and analysed on coded slides by one person.

Statistical analysis. Statistical evaluation was performed by Student's *t*-test [18] and linear correlation.

Results

Table 1 shows the mean concentrations of the biochemical and biological measures in the three groups studied, i.e. in non-exposed non-smokers, non-smokers who had been exposed to environmental tobacco smoke 40 ± 5 h per week, and in cigarette smokers. The biochemical markers, carboxyhaemoglobin and plasma thiocyanate, are widely used, even though they are unspecific for

Table 1. Mean values (and SD) of biological and biochemical measures by the degree of tobacco smoke exposure

	Non-smokers		Smokers		All (n = 22)
	Exposure None (n = 20)	40 h/wk (n = 27)	10-19 cig/d (n = 8)	20-40 cig/d (n = 14)	
Biochemical					
Carboxyhaemoglobin (%)	0.6 (0.2)	0.7 (0.3)	3.3 (1.3)	4.6 (2.6)	4.2*** (2.3)
Plasma thiocyanate (μmol/l)	46 (16)	58** (18)	136 (49)	149 (42)	144*** (45)
Biological					
Leucocyte count (× 10 ⁹ /l)	6.3 (1.3)	8.0* (1.4)	10.0 (3.5)	9.1 (2.8)	9.4*** (3.0)
Sister chromatid exchange (SCEs/cell)*	7.87 (0.68)	7.92 (0.65)	8.58 (1.5)	9.40 (0.77)	9.06* (1.1)
Urinary mutagenicity					
No. positive samples/no. tested	1/17	4/26	4/8	8/11	12/19
Induced revertants/ 25 μl urine concentrate	4.2 (2.2)	4.7 (9.7)	16.4 (15.7)	19.9 (12.7)	17.5*** (8.4)

Non-smokers: n = 14 for non-exposed, n = 20 for exposed; n = 12 for smokers.

* $P < 0.05$; ** $P < 0.01$; *** $P < 0.001$, compared with the mean value of the non-exposed group; t -tailed Student's *t*-test.

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Table 2. Mean plasma and urinary cotinine concentrations in non-exposed non-smokers, non-smokers with occupational exposure to tobacco smoke and smokers

	Plasma (ng/ml)			Urine (ng/ml)		
	Number	Mean	(SD)	Number	Mean	(SD)
<i>Non-smokers</i>						
Non-exposed	20	5.2	(1.5)	20	8.3	(6.7)
Exposed at work	26	10***	(4.0)	27	56***	(37)
<i>Active smokers</i>						
10-19 cig/d	8	219	(87)	8	1452	(539)
20-40 cig/d	14	257	(98)	13	1656	(888)
All	22	246***	(91)	21	1578***	(765)

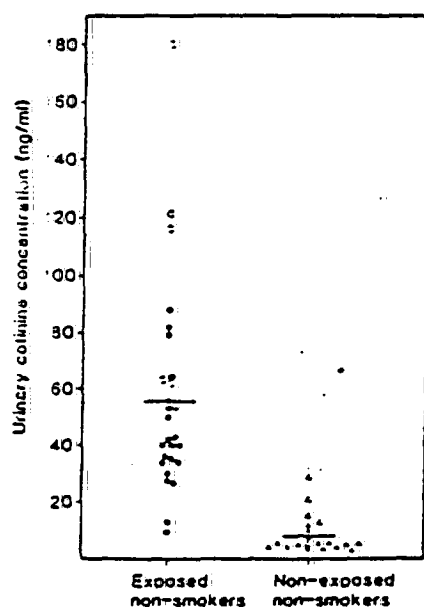
*** $P < 0.001$, compared with the non-exposed group; 2-tailed Student's *t*-test

Fig. 1. Distributions (and means) of cotinine concentrations in urine among non-smokers. ●: non-smokers exposed to environmental tobacco smoke 40 h/week at work; ○: non-smokers exposed at work and at home; ▲: non-smokers with no exposure; Δ: non-smokers with occasional exposure at work

tobacco smoke exposure. The actively smoking waiters and waitresses showed significantly increased levels of carboxyhaemoglobin and thiocyanate concentrations. Also, the mean concentration of plasma thiocyanate in the passively exposed group ($58 \mu\text{mol/l}$) was significantly elevated, when compared with the mean value among the non-exposed non-smokers ($46 \mu\text{mol/l}$; $P < 0.05$; Table 1). However, the mean value for the carboxyhaemoglobin in exposed non-smokers did not deviate from the corresponding value in the non-exposed, non-smoker group.

The mean concentrations of urinary and plasma cotinine for all three study groups are given in Table 2. The different exposure groups showed significantly

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different mean values. The urinary cotinine concentrations for the passively exposed restaurant personnel and for the non-exposed controls are shown in Fig. 1. The ranges for the cotinine concentrations determined in plasma were 83–118 ng/ml, 4.9–20.8 ng/ml and 3.5–8.7 ng/ml for the actively, passively and non-exposed groups; the ranges of cotinine in urine were 480–3810 ng/ml, 9.0–180 ng/ml and 3.0–29 ng/ml, respectively.

A similar trend was obtained for the biological parameters measured (Table 1): the highest values were seen in active smokers, while the mean for the passively exposed group was between the levels for the non-exposed and the actively exposed persons. The mean urinary mutagenic activity, indicating intake and excretion of mutagenic compounds, was increased in smokers, but the exposed non-smokers did not show significantly increased activity as compared with the non-exposed controls. However, the number of positive urine samples was four among the 26 tested passive smoker samples compared with one among the 17 control samples and 12 among the 19 smoker samples. The mean value for total leucocyte count in the passive smokers was found to be above the level observed in the non-exposed group (Table 1: mean for ex-smokers $7.2 \times 10^9/l$, $n = 12$ and for never-smokers $8.6 \times 10^9/l$, $n = 15$, in passive exposure group). Table 1 also gives sister chromatid exchange frequencies analysed in the metaphase chromosomes of cultured lymphocytes. The active smokers had the highest mean frequency, but no difference was observed between the means in the two non-smoker groups.

Discussion

The results of the present study show that non-smokers, who regularly work in environments with heavy smoking, intake clearly detectable amounts of several components of tobacco smoke through passive exposure. The overall level of tobacco-specific markers of intake was somewhat higher than in previous studies based on self-reported estimates of exposure [8]. For urinary cotinine, the mean concentration in exposed non-smokers was 3.5% of the level found in smokers, and the level for plasma cotinine was 4.1% of that observed in smokers; in the non-exposed non-smokers the respective values were 0.5 and 2.1%. The nature and the consistency of the exposure in the restaurant environment, together with the physical activity while working, may have led to increased intake of particulate phase components of tobacco smoke. The active smokers in the present study showed concentrations of biochemical measures comparable with data from the literature [8, 19, 20].

Although no air parameters specific to tobacco smoke were measured in the restaurants, the concentrations of polynuclear aromatic compounds including total polynuclear hydrocarbons (PAHs) and benzo(a)pyrene (BaP) as well as the amount of total particulate matter in the air were determined and found to be relatively high (highest values 167.8 ng/m³, 13.3 ng/m³ and 1.4 mg/m³ for PAHs, BaP and total airborne particulate matter, respectively [21]).

Carbon monoxide, a gas phase component of tobacco smoke, has been found to be associated with subjective irritative symptoms in experimental situ-

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ations at CO concentrations exceeding 2 ppm [22]. Compared with the non-exposed control group, the passive smokers did not show significantly higher level of carboxyhaemoglobin in their blood after 3 to 5 h of work. The COHb levels in both groups of non-smokers were slightly lower than observed previously among urban non-smokers, either in experimental [23, 24] or natural [8, 25-27] settings of passive tobacco smoke exposure: only four passive smokers had a carboxyhaemoglobin level $\geq 1\%$. CO concentration in the air was measured in one of the restaurants and it was at a moderate level (6 to 9 ppm).

The thiocyanate concentration in plasma and involuntary exposure to tobacco smoke have previously been found not to be related [8] or to be only weakly correlated [28]. Still, maternal as well as fetal SCN has been found to be increased in association with passive smoking of the mother [29]. In the present study, SCN in long-term, passive smokers was significantly ($P < 0.05$) at a higher level than in non-smokers with no regular smoke exposure. Among the smokers, plasma thiocyanate concentration was correlated both with the cotinine values in plasma and in urine (P-SCN vs P-cot, $r = 0.43$, $P < 0.05$; P-SCN vs U-cot, $r = 0.48$, $P < 0.05$), but no significant correlation between these parameters was observed among passive smokers or non-exposed controls (among passive smokers: $r = 0.34$, $r = 0.08$, and controls: $r = 0.23$, $r = 0.32$, resp.). Differences in the dietary sources of plasma thiocyanate may influence the results in the non-smoker groups, who have lower levels of tobacco smoke exposure [30, 31]. Both thiocyanate and cotinine are metabolites with relatively long half-lives [31, 32], which means that they rather measure average daily exposure than acute exposure. This makes them suitable for monitoring primarily long-term exposure situations, such as work-related exposure in restaurants.

In the present study we found the overlap in the cotinine values between the active smokers and the passively exposed non-smokers to be negligible. Jarvis et al. [8] estimated the cut-off point for plasma cotinine for discriminating active smokers and non-smokers to be 20 ng/ml. On the basis of this criterion, our passive smoking population was comprised of true non-smokers: only one non-smoker showed a cotinine concentration in plasma that was above the cut-off point. She was a 42-year-old waitress with 20.8 ng/ml cotinine in plasma and 180 ng/ml in urine; but here COHb saturation was only 1.20% and plasma thiocyanate 51 $\mu\text{mol/l}$. These values are lower than found in active smokers.

The biological measures (WBC, SCE) reflecting not only intake of tobacco smoke components, but also biological changes associated with the exposure, were elevated in the active smokers as shown previously [3, 33-35]. Interestingly, according to our preliminary finding, the total leucocyte count appears to be at an intermediate level among the passive smokers in relation to smokers and non-exposed non-smokers. This observation needs to be confirmed in further studies, since the diurnal variation in the white blood cell count [36, 37] may cause bias in the results due to differences in timing of the blood sampling. However, when the data from the exposed samples (active or passive exposure) were pooled, the total leucocyte count correlated with plasma and urinary cotinine values ($r = 0.41$ and $r = 0.45$, $P < 0.01$). It is difficult to evaluate the significance of this unspecific finding. Recent epidemiological observations have suggested that the total leucocyte count may be of prognostic importance.

for coronary heart disease and cancer mortality [38]. The WBC count has also been found to correlate inversely with lung function measurements [39]. In accordance with some earlier results [40, 41], the mean frequencies of sister chromatid exchanges were closely similar in both non-smoker groups, although the extracted air samples from the restaurants were highly active in inducing SCEs when tested in mammalian cell cultures [21]. In experimental situations, passive smokers have shown mutagenicity in urine [14, 42]. In the present study, the passive smokers showed only a trend of higher urinary mutagenicity compared to the non-exposed persons, although the samples of tobacco smoke contaminated air were strongly mutagenic in the bacterial test [21].

The results of the present study support the use of cotinine and/or thiocyanate to measure passive exposure to tobacco smoke. From the intake markers utilized, plasma and urinary cotinine as well as plasma thiocyanate values indicate exposure of the non-smokers to the respective tobacco smoke components in the occupational environment. These indicators thus suggest that exposure to other components of tobacco smoke, including genotoxic ones, may have occurred.

Acknowledgements. We thank Ms. Sinikka Valkonen, Eng. for the analyses of carboxyhaemoglobin and thiocyanate; Ms. Eila Korolainen and Ms. Hilkkka Järventaus, for technical assistance; Ms. Ritva Luukkonen, MSc, for help in statistical analyses; all from the Institute of Occupational Health. We are grateful to the restaurant personnel who participated in the study. This study was supported by the Finnish Medical Board of Health (grant 3.2.5/85) and the Research Council for Environmental Sciences, Academy of Finland (research grant 30/064).

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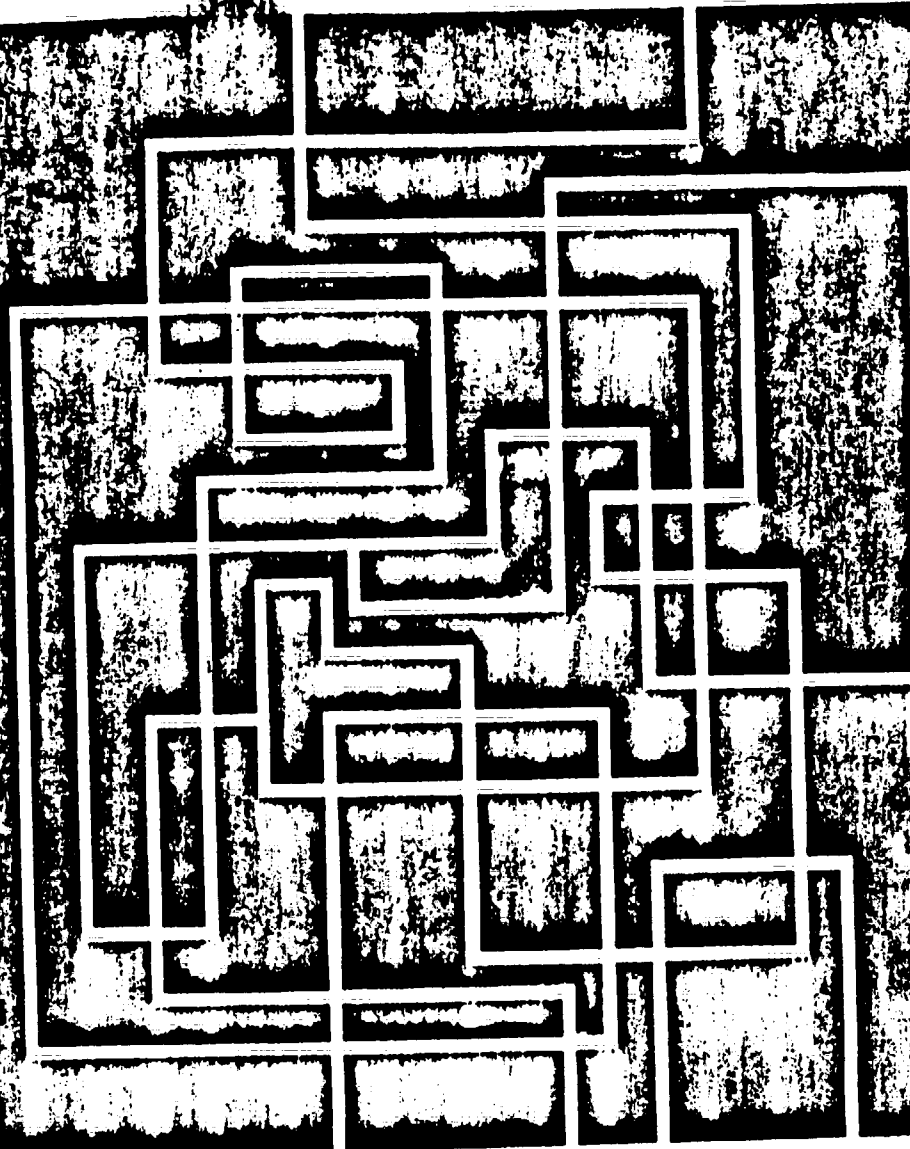
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Received August 18, 1986 / Accepted January 20, 1987

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Toxicology Letters



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TOXICOLOGY LETTERS

*An International Journal for the Rapid Publication of Short Reports
on Biochemical Mechanisms of Mammalian Toxicity*

Proceedings of the International Experimental Toxicology Symposium on Passive Smoking

*held on October 23-25, 1986, University Medical Center of Essen, D-4300
Essen (F.R.G.)*

Guest Editor:
E. Mohtashamipour

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Preface

THE 1986 MEETING ON EXPERIMENTAL TOXICOLOGY OF PASSIVE SMOKING*

The goal of the meeting was to stimulate the exchange of new information among toxicologists and other medical scientists actively involved in experimental studies of the problem of passive smoking.

It has not been more than a few years since the medical profession became concerned again about the health consequences of tobacco-derived environmental air pollution. The stimulating causes of the need for research into such an old problem were the results of some recent epidemiological studies that warned of the cancer risks for individuals passively exposed to tobacco smoke. Although these studies did in fact cause a renewal of clinical research activities, further toxicological experiments were felt needed to foresee the extent of the health consequences before the clinical signs are observed. The hazards caused by involuntary smoking are mainly due to the chemical constitution of the sidestream smoke, which cannot be foreseen when compared with the health consequences caused by active smoking.

We hope the outcome of the meeting on experimental toxicology of passive smoking provides an invaluable basis for the work of those who search for the underlying causes of this health problem.

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*Supported by the German Society for Occupational Medicine (F.R.G.); the Federal Ministry of Youth, Family and Health (F.R.G.) and the Ministry of Science and Research, State of North Rhine-Westphalia (F.R.G.).

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URINARY EXCRETION OF MUTAGENS IN PASSIVE SMOKERS*

(Mutagenicity: urine; cotinine; passive smoking)

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SUMMARY

Six healthy young volunteers with no history of active smoking were asked to keep on their Western diets avoiding the consumption of alcoholic beverages, excess coffee, any sort of medicament, and the known pro- and/or anti-mutagen-containing foods and drinks, 24 h before and during the experiments. They were exposed passively to cigarette smoke produced by 4 habitual smokers in an unventilated 48.6 m³ room for 8 h. The carbon monoxide concentration was 18.85 ± 7.3 ppm during the 8-h exposure.

Frameshift mutagens were isolated from 10-h urine samples using chloroform and were tested for mutagenicity in the *Salmonella*/mammalian microsome assay employing *Salmonella typhimurium* TA98. Although clearly enhanced, no significant mutagenic activity could be found with 25 ml equivalent urine/plate after passive exposure to cigarette smoke. The weak mutagenicities found were highly significant when 50 ml equivalent urine/plate was tested. No direct correlation was observed between urine mutagenicity and the urinary cotinine concentration. The results obtained are discussed with reference to inconsistent reports in the literature concerning the mutagenicity of urine after passive smoking.

INTRODUCTION

Inconsistent evidence exists in the literature concerning the recovery of mutagens excreted in urine after passive smoking. Bos et al. [1] reported, for the first time, that 12-h urine of nonsmokers staying in a smoky room for 6 h was mutagenic. In contrast, the 1985 report of Sorsa et al. [2] indicated no significant (although suggestive) difference between urine mutagenicity before and after passive smoking (their subjects were habitual smokers after a 48-72 h smoking cessation). The very recent paper of Scherer et al. [3] indicates non-mutagenicity of the urine of

* Presented at the International Experimental Toxicology Symposium on Passive Smoking, October 23-25, 1986, Essen (F.R.G.).

nonsmokers who were kept on a diet low in polycyclic aromatic hydrocarbons but smoked passively for 8 h.

To clarify the reason(s) for the presence of these discrepancies in reports, we performed an experiment on human subjects who smoked passively while being on a semi-controlled diet.

MATERIALS AND METHODS

Volunteers

6 healthy volunteers, 1 male and 5 female, with no smoking experience (mean age: 24.7 ± 1.5 years; mean body weight: 56.5 ± 5.2 kg) were asked to keep to normal Western diets excluding the consumption of alcoholic beverages, any sort of medication (except contraceptives), and the known pro- and/or anti-mutagens such as grilled or fried meats [4,5], ascorbic acid, tocopherol or retinol [6-8] or the beverages that contain excess amounts of these vitamins, 24 h before and during the experiments. Volunteers were also requested to avoid staying in smoky places where smokers smoke from 24 h before the experiments began. None had an industrial occupation, neither were they actively exposed to industrial chemicals.

Smoke exposure

4 habitual smokers began to smoke in a room of 48.6 m³ about half an hour before the nonsmokers were admitted into that experimental room (CO was about 15 ppm at the time of admission). All the volunteers sat around a table (one smoker between two nonsmokers) for an 8-h exposure during which 88 cigarettes of the same type were smoked by smokers. The cigarettes were a blond commercial filtered type of regular size (tar yield 13 mg; nicotine yield: 0.9 mg). No ventilator was installed in the room and the door and windows were kept closed during the experiment. The volunteers were allowed to leave the room repeatedly to collect their urine. Leaving the room for a rest was not allowed. They had the same meal and soft drinks during the experiment.

Carbon monoxide determination

CO was determined electrochemically using Ecolyzer 2400 (Energetics Science Inc., Elmsford, NY).

Collection of urine samples

10-h samples of the control day were collected on the weekend avoiding the first morning urine. On the day of exposure, any amount of urine that could be collected before entering the experimental room was discarded. The volunteers collected their urine during the 8-h exposure and the following 2 h when they just walked in a park to get some fresh air. The total 10-h samples were kept frozen at -20°C prior to extraction.

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Cotinine determination

The measurement of cotinine in urine was done according to the method of Stehlik et al. [9] using capillary gas chromatography at the following conditions:

column:	quartz capillary WG 11, 30m, \emptyset 0.32 mm, film thickness 0.2 μ m.
carrier gas:	nitrogen 0.5 bar
split ratio:	1:2.3
detector:	nitrogen-sensitive
gas flows	hydrogen 1 ml/min, nitrogen 30 ml/min, air 10 ml/min, septum purge 1 ml/min
temperature:	injector 280°C, detector 280°C, oven program: 3 min 80°C; 80–240°C at 10°C/min, held 6 min; 240–250°C at 10°C/min, held 10 min
injection volume:	1 μ l
time/injection:	40 min

Mutagenicity assays

Urine samples were extracted with chloroform using the method described previously [10]. The extracts were tested for mutagenicity in the *Salmonella*/mammalian microsome assay using *S. typhimurium* TA98 as described elsewhere [11]. Liver homogenates were prepared after induction of liver enzymes by a single i.p. injection of Aroclor 1254 (in Mazola oil) into each 180–200 g male Sprague-Dawley Bio/1 rat [11]. The assays were performed in triplicate experiments per dose.

RESULTS

The CO concentration at the beginning of the experiments when the nonsmokers entered the experimental room was about 15 ppm. Later on, the CO concentration throughout the 8-h exposure to cigarette smoke was 18.85 ± 7.3 ppm. Non-mutagenicities of the urine samples collected before passive smoking are documented in Table I. A fairly clear dose-response relationship was detected when the urine samples collected after passive exposure to cigarette smoke were tested for mutagenicity (Table I). In this connection, although 25 ml equivalent urine per plate clearly enhanced the number of revertants, using the standard of Ames et al. [11] for interpretation of the mutagenicity data, no doubling of the revertants was observed. Doubling this latter amount of urine/plate resulted in more than 100% increase in the mutation frequency of the tester strain (Table I).

Exceptionally, testing the urine of volunteer E did not result in detection of a significant mutagenic activity, although cotinine was found in her urine in a high quantity (Table I).

Comparing the urine concentrations of cotinine before and after passive smoking, the urine samples collected after exposure contained 1.1–5.4-fold more cotinine than

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The samples were analysed by gas-liquid chromatography-mass spectrometry and were subjected to thin-layer chromatography to be tested for mutagenicity. The results were tested by the Salmonella/microsome assay employing *Salmonella typhimurium* TA98 in the presence of NADPH-generating system and *Ames* induced rate liver homogenates. No significant differences were observed.

Concentration		Mean number of bacteria/plate			
Before exposure		Before exposure		After exposure	
Urine		Urine		Urine	
Concentration	Urine	Before exposure	After exposure	Before exposure	After exposure
0.001	0.001	100 ± 10	100 ± 10	100 ± 10	100 ± 10
0.002	0.002	100 ± 10	100 ± 10	100 ± 10	100 ± 10
0.005	0.005	100 ± 10	100 ± 10	100 ± 10	100 ± 10
0.01	0.01	100 ± 10	100 ± 10	100 ± 10	100 ± 10
0.02	0.02	100 ± 10	100 ± 10	100 ± 10	100 ± 10
0.05	0.05	100 ± 10	100 ± 10	100 ± 10	100 ± 10
0.1	0.1	100 ± 10	100 ± 10	100 ± 10	100 ± 10
0.2	0.2	100 ± 10	100 ± 10	100 ± 10	100 ± 10
0.5	0.5	100 ± 10	100 ± 10	100 ± 10	100 ± 10
1.0	1.0	100 ± 10	100 ± 10	100 ± 10	100 ± 10
2.0	2.0	100 ± 10	100 ± 10	100 ± 10	100 ± 10
5.0	5.0	100 ± 10	100 ± 10	100 ± 10	100 ± 10
10.0	10.0	100 ± 10	100 ± 10	100 ± 10	100 ± 10
20.0	20.0	100 ± 10	100 ± 10	100 ± 10	100 ± 10
50.0	50.0	100 ± 10	100 ± 10	100 ± 10	100 ± 10
100.0	100.0	100 ± 10	100 ± 10	100 ± 10	100 ± 10
200.0	200.0	100 ± 10	100 ± 10	100 ± 10	100 ± 10
500.0	500.0	100 ± 10	100 ± 10	100 ± 10	100 ± 10
1000.0	1000.0	100 ± 10	100 ± 10	100 ± 10	100 ± 10
2000.0	2000.0	100 ± 10	100 ± 10	100 ± 10	100 ± 10
5000.0	5000.0	100 ± 10	100 ± 10	100 ± 10	100 ± 10
10000.0	10000.0	100 ± 10	100 ± 10	100 ± 10	100 ± 10
20000.0	20000.0	100 ± 10	100 ± 10	100 ± 10	100 ± 10
50000.0	50000.0	100 ± 10	100 ± 10	100 ± 10	100 ± 10
100000.0	100000.0	100 ± 10	100 ± 10	100 ± 10	100 ± 10
200000.0	200000.0	100 ± 10	100 ± 10	100 ± 10	100 ± 10
500000.0	500000.0	100 ± 10	100 ± 10	100 ± 10	100 ± 10
1000000.0	1000000.0	100 ± 10	100 ± 10	100 ± 10	100 ± 10
2000000.0	2000000.0	100 ± 10	100 ± 10	100 ± 10	100 ± 10
5000000.0	5000000.0	100 ± 10	100 ± 10	100 ± 10	100 ± 10
10000000.0	10000000.0	100 ± 10	100 ± 10	100 ± 10	100 ± 10
20000000.0	20000000.0	100 ± 10	100 ± 10	100 ± 10	100 ± 10
50000000.0	50000000.0	100 ± 10	100 ± 10	100 ± 10	100 ± 10
100000000.0	100000000.0	100 ± 10	100 ± 10	100 ± 10	100 ± 10
200000000.0	200000000.0	100 ± 10	100 ± 10	100 ± 10	100 ± 10
500000000.0	500000000.0	100 ± 10	100 ± 10	100 ± 10	100 ± 10
1000000000.0	1000000000.0	100 ± 10	100 ± 10	100 ± 10	100 ± 10
2000000000.0	2000000000.0	100 ± 10	100 ± 10	100 ± 10	100 ± 10
5000000000.0	5000000000.0	100 ± 10	100 ± 10	100 ± 10	100 ± 10
10000000000.0	10000000000.0	100 ± 10	100 ± 10	100 ± 10	100 ± 10
20000000000.0	20000000000.0	100 ± 10	100 ± 10	100 ± 10	100 ± 10
50000000000.0	50000000000.0	100 ± 10	100 ± 10	100 ± 10	100 ± 10
100000000000.0	100000000000.0	100 ± 10	100 ± 10	100 ± 10	100 ± 10
200000000000.0	200000000000.0	100 ± 10	100 ± 10	100 ± 10	100 ± 10
500000000000.0	500000000000.0	100 ± 10	100 ± 10	100 ± 10	100 ± 10

Spontaneous mutation frequency: 34.0×10^{-6}

the samples collected before (Table I). An exception was the urine of subject A, which contained more cotinine before than after exposure. In contrast, only the urine collected after exposure was mutagenic.

DISCUSSION

Controversies are seen among the results of various authors [1-3], which do not permit a conclusion as to whether urine of passive smokers is mutagenic. Our results indicate that the inconsistency of the reports is possible due to some methodical difficulties of extraction and testing procedures and/or the experimental designs. Comparing the results of our mutagenicity assays with those of the cotinine measurements, no clear correlation can be found between urine mutagenicity and the enhancement of cotinine in urine after passive exposure to cigarette smoke.

Our data, in agreement with the findings of Bos et al. [1], indicate that detectable mutagens are excreted in urine after passive smoking. The mutagenicities found with the urine of passive smokers (Table I; last column) are comparable with those of nonsmokers after active smoking of 4 or 5 cigarettes [10]. Thus the previous negative reports of other authors [2,3] might hint at minute shortcomings of the experimental designs such as testing low volumes of urine [3] or admitting smoke-experienced volunteers to play the unexperienced nonsmokers' roles during a short smoking cessation [2]. Urinary excretion of mutagens depends on a variety of factors, the diet being one of them [5,12]. This latter includes both pro- and anti-mutagens [4-8,12-14]. A simple difficulty might arise in the recovery of urinary mutagens when the test person's consumption of dietary anti-mutagens is disregarded. Additionally, endogenous formation or detoxification of carcinogenic/mutagenic compounds should not be disregarded as the contributing factors.

ACKNOWLEDGEMENTS

Out thanks are due to Karin Wöhrmeyer and Birgit Wiethaup for their invaluable technical assistance.

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A genotoxic assessment of environmental tobacco smoke using bacterial bioassays

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(Received 25 June 1987)

(Revision received 12 October 1987)

(Accepted 16 October 1987)

Keywords: Environmental tobacco smoke; *Salmonella typhimurium*; Indoor environments; Chemistry; Semi-volatiles; Monitoring

Summary

Recently, the National Research Council in the U.S.A. stated that laboratory studies of environmental tobacco smoke (ETS) should be important in identifying ETS carcinogens, their concentrations in typical daily environments, and in understanding how these compounds contribute to ETS dose-response relationships. This paper demonstrates that integrated chemical and bacterial mutagenicity information can be used to identify ETS genotoxicants, monitor human exposure, and make comparative assessments. Approximately 1/3 of the ETS constituents for which there is quantitative analytical chemistry information also have associated genotoxicity information. For example, 11 of the quantitated compounds are animal carcinogens. Work presented in this paper demonstrates that both the nonparticle-bound semi-volatile and the particulate-bound organic material contain bacterial mutagens. These ETS organics give an equivalent of ~ 86 000 revertants per cigarette. In addition, this article summarized efforts to estimate ETS bacterial mutagenicity, to use bacterial tests for the monitoring of ETS-impacted indoor environments, and to use bacterial assays for the direct monitoring of human exposure.

Environmental tobacco smoke (ETS) is the total tobacco smoke found in an environment and includes both sidestream cigarette smoke and the exhaled tobacco smoke of the smokers within the environment. The first suggestion that environmental tobacco smoke (ETS) could have detrimental health effects was a medical case report published by Rosen and Levy in 1950. This report

concluded that an infant's severe asthmatic symptoms were directly related to the mother's smoking of tobacco products. It was not until 31 years later that the results from epidemiological studies of passive smoking and lung cancer were available. Three studies were published in 1981. Based on a population of 91 540 nonsmoking Japanese housewives, Hirayama (1981) reported that the wives of heavy smokers (> 20 cigarettes/day) had 2.4 times the risk of developing lung cancer as the nonsmoking wives of nonsmokers. Trichopoulos et al. (1981) reported a slightly larger risk for nonsmoking Greek women whose husbands smoked

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0165-1218/89/\$03.50 © 1989 Elsevier Science Publishers B.V. (Biomedical Division)

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more than 20 cigarettes per day. Although Garfinkel (1981) found a small (10–30%) increase in mortality associated with passive smoking, his study of 469 000 nonsmokers did not detect a statistically significant increase in risk of developing lung cancer. Since that time, a number of other epidemiologic studies have emerged (National Research Council, 1986). The National Research Council (1986) concluded that the summary estimate of increased risk of lung cancer ranges from 10% to 34%. They also concluded that although bias may contribute to the results, the best estimate at present for increased adjusted risk of lung cancer to nonsmokers due to passive exposure is approximately 25%. In identifying needed scientific information, the National Research Council (1986) stated: 'Laboratory studies should be important in determining the carcinogenic constituents of ETS and their concentrations in typical daily environments and in facilitating understanding of possible dose-responsive relationships.' The purpose of this paper is to partially fulfill this need by integrating chemical and bacterial genetic bioassay information concerning sidestream and indoor air (IA) tobacco smoke.

Materials and methods

Integrating known chemical and genotoxicity information for sidestream cigarette smoke

Although it is expected that most of the compounds found in mainstream tobacco smoke (MS) would also be found in sidestream smoke (SS), the purpose of this effort was to extract from the literature compounds identified specifically in SS and to associate this information with presently available genotoxicity information. As part of an earlier effort to identify airborne compounds and genotoxicity information (Graedel et al., 1986), confirmed compounds found in tobacco smoke and indoor air were cataloged. This summary provided the baseline information for this tabulation. In addition, an on-line *Chemical Abstracts* search was used to locate relevant papers published since January, 1983. The compounds identified in these papers were summarized, and the MS, SS, and/or indoor air (IA) concentrations were tabulated. Where available, the bioassay summary information as reported by Graedel et al. (1986) was

paired with the identified chemicals. All computer literature searches were kindly supplied by the Resource Information Center, U.S. Environmental Protection Agency, Research Triangle Park, NC (U.S.A.) via Ms. Libby Smith. Although the search periods covered a time period extending to December 1986, the information is not meant to be exhaustive but representative of the available literature.

Methods for determining the Salmonella mutagenicity of organic extracts from sidestream tobacco smoke particles

After being lit, a generic U.S. brand of filter cigarettes was allowed to burn within a 0.04-m³ Plexiglass® chamber into which filtered air was allowed to enter. The generated SS was continuously exhausted at a rate of 0.03 m³/min and collected on a 142-mm Teflon®-coated glass-fiber filter. Sample preparation was done in the manner reported by Morin et al. (1987). The method can be summarized as follows. All filter samples were extracted using 2 15-min sonications (Constant Temperature, Sonicator® waterbath sonicator, Bay Shore, NY) using either dichloromethane, methanol, or acetone as the solvent. Extracted samples were concentrated to 5–10 ml using rotatory evaporation. Samples were then transferred into 15-ml volumetric tubes and concentrated to 1–2 ml using dry nitrogen. After adding 15 ml of dimethyl sulfoxide (DMSO), the remaining solvent was removed using the nitrogen purge method. Negative controls were prepared in the same manner using filters not containing ETS particles. The solvent-exchanged samples were tested using the *Salmonella typhimurium* plate incorporation assay as previously described (Ames et al., 1975; Claxton et al., 1987).

Direct comparisons of mainstream and sidestream tobacco smoke bacterial mutagenicity

In order to compare the total genotoxic potential of MS and SS it was necessary to bioassay the particle-bound, semi-volatile, and volatile compounds emitted. In order to accomplish this, separate trapping trains for MS and SS were used. Each train consisted of an ethanol bubbler solvent trap, a sand trap, and a liquid-nitrogen cold trap in sequence (Monteith et al., 1986). Cigarette sam-

ples were generated using a standard corvette for the 30 cigarette puff duration. Mainstream MS trapping cigarette smoke was collected in a Tedlar® bag. Air from the SS trapping primarily sidestream smoke was collected prior to the sand trap. The sand trap was bioassayed in the same version of the assay as the MS (1975). Prior to use, while vials were being prepared, rpm. Tests were performed using Aroclor-1254 homogenate. The homogenate was transferred to a standard bioassay test were conducted (1975).

Results and discussion

When one compares the organic emissions and varied approaches in the introduction of ETS (Hirayama, Garfinkel, 1986). Although statistical likelihood with ETS, the answer more and human health not identify variations are careful of toxicants to determine type used, cigarette rates, the health implications logical data available to

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ples were generated using a smoking machine. The standard conditions were 1 puff/min for each of the 30 cigarettes (1R3 Kentucky Reference), a puff duration of 2 s, and a puff volume of 35 ml. Mainstream smoke was pulled directly into the MS trapping train. In order to collect the SS, the cigarette smoking machine was enclosed within a Tedlar[®] bag which had a total volume of 0.12 m³. Air from the bag was continuously drawn into the SS trapping trains. The ethanol bubbler collected primarily semi-volatile compounds, the sand trap collected primarily particulate matter, and the cold trap collected any remaining organic compounds. The sand trap and semi-volatile concentrates were bioassayed in sealed dram vials using a modified version of the preincubation protocol of Yahagi et al. (1975). Preincubation was for 15 min at 37°C while vials were shaken by a rotary shaker at 60 rpm. Tests were done using 500 µl/plate of 5% Aroclor-1254-induced male Syrian hamster liver homogenate. Volatiles from the cold trap were transferred as a gas to Tedlar[®] bags containing standard bioassay plates. All other aspects of the test were conducted as described by Ames et al. (1975).

Results and discussion

When one evaluates the hazard of a complex organic emission such as ETS, there are multiple and varied approaches that one may use. As stated in the introduction, several researchers have assessed ETS through epidemiological efforts (Hirayama, 1981; Trichopoulos et al., 1981; Garfinkel, 1981; National Research Council, 1986). Although such studies can demonstrate the statistical likelihood of increased risk associated with ETS, these studies were not designed to answer more specific questions concerning ETS and human health. For example, these studies do not identify which constituents within ETS emissions are carcinogens, mutagens, and other types of toxicants. Epidemiology, therefore, is unlikely to determine whether or not changes in tobacco type used, cigarette design, tar values, room ventilation rates, etc. will produce major changes in the health impact of ETS. When human epidemiological data are unavailable and unlikely to be available to answer such relevant issues, one can

sometimes use whole animal studies (Stara and Kello, 1979). However, since whole animal studies for carcinogenesis and heritable mutations are expensive, lengthy processes, they too are unlikely to be useful for answering many relevant questions. Short-term test data, especially when associated with quantitative data on analytical chemistry, can be used to make comparative assessments, to identify genotoxicants, and to monitor human exposure.

After identification and quantification of the individual constituents of the mixture, the known toxicological properties of each constituent can be related to the source, for example ETS. The total potential impact of a source can then be estimated by summing the activity of the known constituents or by using some form of toxicological modeling or scaling.

Approximately 10% of the more than 3800 compounds found in MS have been identified in ETS. Table 1 lists over 100 constituents found in ETS and in MS and IA samples for which there is quantitative analytical information. The relative concentrations of the constituents vary according to whether they are measured as MS, SS, or IA components. The reasons (e.g., combustion temperature, oxygen levels, etc.) for differences in concentrations of organic components in MS and SS have been discussed previously (Sakuma et al., 1983, 1984; Baker, 1981, 1982; Klus and Kutin, 1982; IARC, 1968; National Research Council, 1986).

Table 2 lists the genotoxicity information (Graedel et al., 1986; Nesnow et al., 1987) for the compounds in Table 1. Approximately 1/3 of the compounds in Table 1 have associated genotoxicity information. Eleven of these compounds are animal carcinogens (Nesnow et al., 1987). Nineteen of the compounds are positive in at least 1 bioassay, and 10 are positive in the Salmonella bioassay (Graedel et al., 1986). This type of information provides a qualitative evaluation of potential human health impacts. For comparative risk analysis methods (Lewtas, 1985), the amount of each constituent can be associated with the bioassay activity of each constituent in order to provide a crude means of comparison between sources or components of interest. For example, using concentrations of constituents in SS and their known

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TABLE 1

CONCENTRATIONS OF COMPOUNDS ASSOCIATED WITH MAINSTREAM AND SIDESTREAM TOBACCO SMOKE AND INDOOR AIR POLLUTED WITH TOBACCO SMOKE ^a

Compound	Sample type	Concentration range			Ref. ^b
		Low	High	Units	
Acetanide	MS	70.00	111.00	μg/cig	25
Acetamide	SS	86.00	156.00	μg/cig	25
Acetic acid	MS	333.00	809.00	μg/cig	23
Acetic acid	MS	272.00	475.00	μg/cig	25
Acetic acid	SS	1241.00	2187.00	μg/cig	23
Acetic acid	SS	695.00	1148.00	μg/cig	25
Acrolein	IA	0.90	1.30	ppm	2
Acrolein	IA	0.02	0.12	mg/m ³	3
Acrolein	IA	6.00	10.00	ppm	12
Acrolein	IA	0.01	0.19	mg/m ³	19
Acrolein	SS	50.00	70.00	ppm	2
Acrolein (gas only + people)	IA	130.00	190.00	μg/m ³	19
Acrolein (people absent)	IA	119.00	133.00	μg/m ³	19
Acrolein (people present)	IA	10.00	48.00	μg/m ³	19
Acrolein control air	IA	0.00	5.00	ppm	12
Aldehydes (gas only + people)	IA	1290.00	1350.00	μg/m ³	19
Aldehydes (generic)	IA	0.39	1.37	mg/m ³	19
Aldehydes (people absent)	IA	1100.00	1370.00	μg/m ³	19
Aldehydes (people present)	IA	391.00	622.00	μg/m ³	19
Alkoxy radicals	MS	8.00 × 10 ¹⁵ spins/c			22
Alkoxy radicals	SS	6.00 × 10 ⁶ spins/c			22
Ammonia	MS	79.40	131.00	μg/cig	9
Ammonia	MS	95.30	163.00	μg/g smoked	9
Ammonia (cigars)	MS	30.50	322.00	μg/g smoked	9
Ammonia (cigars)	MS	148.00	288.00	μg/product	9
Ammonia	SS	5.14	5.77	mg/cig	9
Ammonia	SS	6.11	7.18	mg/g smoked	9
Ammonia (cigars)	SS	6.98	106.00	mg/cig	9
Ammonia (cigars)	SS	9.34	20.50	mg/g smoked	9
Anatabine	MS	2.40	20.10	μg/cig	24
Anatabine	SS	0.00	2.40	μg/cig	24
Anthanthrene	IA			Qual (ng/m ³)	1
Anthanthrene	IA	3.00		ng/m ³	15
Anthanthrene	MS	22.00		ng/cig	14
Anthanthrene	SS	39.00		ng/cig	14
Anthracene	IA			Qual (ng/m ³)	1
Anthracene	MS, P	23.60		ng/cig	16
Anthracene	MS, V	0.10		ng/cig	16
Anthracene	SS, P	670.00		ng/cig	16
Anthracene	SS, V	40.00		ng/cig	16
Benz[a]anthracene	IA			Qual (ng/m ³)	1
Benz[a]anthracene	MS, P	13.30		ng/cig	16
Benz[a]anthracene	MS, V	0.09		ng/cig	16
Benz[a]anthracene	SS, P	201.00		ng/cig	16
Benz[a]anthracene	SS, V	2.50		ng/cig	16

TABLE 1 (co

Compound

Benz[e]acena[...]

Benzene

Benzene (brea)

Benzene (hom:

BRITISH (1901)

Второй этап — это

Benz[a]fluorene

2. *1999*

Benzol(a)pyren

Benzolaldehyd

Вспомогательный

Hexachloropyren

Неокий и Юрген

Benz(a)pyren

111

Հետզոյ Ի խորհու

Benard's t test

Benzylb/c1flu

44-38861-1000

Ben-10(b/j/l)

benzo[h/j/k]

Environ Monit Assess

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 100. **Список даты**

Benzo(e)pyrene

Brucella pyrenae

2,4,6-triphenylpyrene
fluoride

benzofuran

Ann. de l'Épave

Amur (ghu) Nuo

Law. 4841/1969

100% Satisfaction
 Guaranteed

W.D. White Paper

TABLE 1 (continued)

Compound	Sample type	Concentration range		Units	Ref. ^b
		Low	High		
Benz[<i>e</i>]acenaphthylene	IA			Qual (ng/m ³)	1
Benzene	IA	0.05	0.15	mg/m ³	3
Benzene (breath. non-smokers)	IA	2.50		μg/m ³	30
Benzene (breath. smokers)	IA	16.00		μg/m ³	30
Benzene (homes. non-smokers)	IA	4.40	9.20	μg/cm ³	30
Benzene (homes. smokers)	IA	4.80	16.00	μg/m ³	30
Benzo[<i>a</i>]fluorene	IA			Qual (ng/m ³)	1
Benzo[<i>a</i>]fluorene	IA	39.00		ng/m ³	15
Benzo[<i>a</i>]fluorene	MS	184.00		ng/cig	14
Benzo[<i>a</i>]fluorene	SS	751.00		ng/cig	14
Benzo[<i>a</i>]pyrene	IA			Qual (ng/m ³)	1
Benzo[<i>a</i>]pyrene	IA	7.10	21.70	ng/m ³	11
Benzo[<i>a</i>]pyrene	IA	6.20	144.00	ng/m ³	13
Benzo[<i>a</i>]pyrene	IA	22.00		ng/m ³	15
Benzo[<i>a</i>]pyrene	MS	44.00		ng/cig	14
Benzo[<i>a</i>]pyrene	MS, P	10.90		ng/cig	16
Benzo[<i>a</i>]pyrene	MS, V	0.08		ng/cig	16
Benzo[<i>a</i>]pyrene	SS	199.00		ng/cig	14
Benzo[<i>a</i>]pyrene	SS, P	103.00		ng/cig	16
Benzo[<i>a</i>]pyrene	SS, V	0.48		ng/cig	16
Benzo[<i>a</i>]pyrene control	IA	0.00	0.69	ng/m ³	11
Benzo[<i>b</i>]naphtho[2,1- <i>d</i>]thiophene	MS, P	2.80		ng/cig	16
Benzo[<i>b</i>]naphtho[2,1- <i>d</i>]thiophene	MS, V	0.21		ng/cig	16
Benzo[<i>b</i>]naphtho[2,1- <i>d</i>]thiophene	SS, P	50.00		ng/cig	16
Benzo[<i>b</i>]naphtho[2,1- <i>d</i>]thiophene	SS, V	1.10		ng/cig	16
Benzo[<i>b</i> / <i>c</i>]fluorene	MS	69.00		ng/cig	14
Benzo[<i>b</i> / <i>c</i>]fluorene	SS	251.00		ng/cig	14
Benzo[<i>b</i> / <i>j</i> / <i>k</i>]fluoranthene	IA	35.00		ng/m ³	15
Benzo[<i>b</i> / <i>j</i> / <i>k</i>]fluoranthene	MS	49.00		ng/cig	14
Benzo[<i>b</i> / <i>j</i> / <i>k</i>]fluoranthene	SS	260.00		ng/cig	14
Benzo[<i>e</i>]fluorene	IA			Qual (ng/m ³)	1
Benzo[<i>e</i>]pyrene	IA			Qual (ng/m ³)	1
Benzo[<i>e</i>]pyrene	IA	18.00		ng/m ³	15
Benzo[<i>e</i>]pyrene	IA	3.30	23.40	ng/m ³	20
Benzo[<i>e</i>]pyrene	MS	25.00		ng/cig	14
Benzo[<i>e</i>]pyrene	MS, P	6.70		ng/cig	16
Benzo[<i>e</i>]pyrene	MS, V	0.13		ng/cig	16
Benzo[<i>e</i>]pyrene	SS	135.00		ng/cig	14
Benzo[<i>e</i>]pyrene	SS, P	75.00		ng/cig	16
Benzo[<i>e</i>]pyrene	SS, V	0.74		ng/cig	16
Benzo[<i>e</i>]pyrene control	IA	3.00	5.10	ng/m ³	20
Benzo[<i>ghi</i>]fluoranthene	IA			Qual (ng/m ³)	1
Benzo[<i>ghi</i>]perylene	IA			Qual (ng/m ³)	1
Benzo[<i>ghi</i>]perylene	IA	17.00		ng/m ³	15
Benzo[<i>ghi</i>]perylene	MS	39.00		ng/cig	14
Benzo[<i>ghi</i>]perylene	MS, P	7.10		ng/cig	16
Benzo[<i>ghi</i>]perylene	MS, V	0.09		ng/cig	16
Benzo[<i>ggi</i>]perylene	SS	98.00		ng/cig	14
Benzo[<i>ghi</i>]perylene	SS, P	41.00		ng/cig	16
Benzo[<i>ghi</i>]perylene	SS, V	0.62		ng/cig	16

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TABLE 1 (continued)

Compound	Sample type	Concentration range		Units	Ref. ^b
		Low	High		
Benzofluoranthenes (b + j + k)	MS, P	20.50		ng/cig	16
Benzofluoranthenes (b + j + k)	MS, V	0.22		ng/cig	16
Benzofluoranthenes (b + j + k)	SS, P	196.00		ng/cig	16
Benzofluoranthenes (b + j + k)	SS, V	1.38		ng/cig	16
Benzoic acid	MS	14.00	28.00	μg/cig	23
Benzoic acid	SS	12.00	23.00	μg/cig	23
Benzoic acid, <i>m</i> -hydroxy-	MS	8.00	64.00	μg/cig	23
Benzoic acid, <i>m</i> -hydroxy-	SS	3.00	15.00	μg/cig	23
Benzonitrile	MS	5.00	6.00	μg/cig	25
Benzonitrile	SS	33.00	57.00	μg/cig	25
Bipyridyl, 2, 3'-	MS	9.90	27.40	μg/cig	24
Bipyridyl, 2, 3'-	SS	20.00	73.00	μg/cig	24
Bipyridyl, 5-methyl-2, 3'-	MS	6.60	14.70	μg/cig	24
Bipyridyl, 5-methyl-2, 3'-	SS	6.00	14.00	μg/cig	24
Butyrolactone, gamma-	SS	40.00	103.00	μg/cig	25
Butyrolactone, gamma-	MS	11.00	22.00	μg/cig	25
Carbon monoxide	1A	2.00	23.00	ppm	3
Carbon monoxide	1A	0.00	1.20	ppm	31
Carbon monoxide (gas only + people)	1A	23.00	26.00	ppm	19
Carbon monoxide (people absent)	1A	21.00	25.00	ppm	19
Carbon monoxide (people present)	1A	18.00	22.00	ppm	19
Carbon monoxide (people present)	1A	3.70	4.20	ppm	28
Carbon monoxide control	1A	0.00	15.00	ppm	3
Carbon monoxide control	1A	0.00	0.50	ppm	31
Carbon monoxide (artificial cond.)	1A	8.00	16.00	ppm	29
Carbon monoxide (natural conditions)	1A	9.00		ppm	29
Carbon, total	1A	207.00		μg/m ³	27
Carbon, elemental	1A	11.90		μg/m ³	27
Carbon, organic	1A	195.00		μg/m ³	27
Carboxyhemoglobin (blood, passive)	1A	0.55		%	28
Carboxyhemoglobin (blood, smoker)	1A	3.38		%	28
Carboxyhemoglobin (blood, no smoking)	1A	0.57		%	28
Catechol	MS	148.00	362.00	μg/cig	23
Catechol	SS	138.00	292.00	μg/cig	23
Catechol, 2-methyl-	MS	6.00	13.00	μg/cig	23
Catechol, 2-methyl-	SS	8.00	21.00	μg/cig	23
Catechol, 3-methyl-	MS	31.00	62.00	μg/cig	23
Catechol, 3-methyl-	SS	24.00	47.00	μg/cig	23
Catechol, 4-ethyl-	MS	27.00	102.00	μg/cig	23
Catechol, 4-ethyl-	SS	19.00	68.00	μg/cig	23
Catechol, 4-methyl-	SS	25.00	55.00	μg/m ³	23
Catechol, 4-methyl-	MS	29.00	80.00	μg/cig	23
Catechol, 4-vinyl-	MS	23.00	113.00	μg/cig	23
Catechol, 4-vinyl-	SS	7.00	40.00	μg/cig	23
Catechols (all catechols)	MS	25.00	328.00	μg/cig	4
Catechols (all catechols)	SS	88.00	212.00	μg/cig	4
Coronene	1A	0.50	1.20	ng/m ³	20
Coronene control	1A	1.00	2.80	ng/m ³	20

TABLE 1 (CO

Compound

Cocaine (plas)
Counine (plas)
Cronine (plas)

Cresol, m-
Cresol, m-
Cresol, m-
Cresol, m-
Cresol, o-
Cresol, o-
Cresol, p-
Cresol, p-
Cresol, p-

Cyclopentenon
Cyclopentenon
Cyclopentenon:
Cyclopentenon:
Cyclopentenone
Cyclopentenone
Cyclopentenone
Cyclopentenone

Lithraea, f. jani
Lithraea, f. jani
Lithraea, f. jani

Bibenzene (b)
Bibenzene (b)
Bibenzene (b)
Bibenzene (b)

Ethylenether
Ethylenether

Phenanthrene
Phenanthrene
Phenanthrene
Phenanthrene
Phenanthrene
Phenanthrene

Formaldehyde
Formaldehyde
Formaldehyde:

Formic acid
Formic acid

Formylaldehyde, 2-
Formylaldehyde, 2-
Formylaldehyde, 3-m
Formylaldehyde, 3-m

Formyl alcohol
Formyl alcohol

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TABLE 1 (continued)

Compound	Sample type	Concentration range		Units	Ref. ^b
		Low	High		
Cotinine (plasma, non-smoker)	IA	1.40		ng/ml	28
Cotinine (plasma, passive smoker)	IA	2.10		ng/ml	28
Cotinine (plasma, smoker)	IA	52.40		ng/ml	28
Cresol, <i>m</i> -	MS	11.00	18.00	μg/cig	23
Cresol, <i>m</i> -	MS	17.00	26.00	μg/cig	25
Cresol, <i>m</i> -	SS	13.00	24.00	μg/cig	23
Cresol, <i>m</i> -	SS	18.00	34.00	μg/cig	25
Cresol, <i>o</i> -	MS	13.00	19.00	μg/cig	23
Cresol, <i>o</i> -	SS	14.00	24.00	μg/cig	23
Cresol, <i>p</i> -	MS	30.00	37.00	μg/cig	23
Cresol, <i>p</i> -	MS	32.00	47.00	μg/cig	25
Cresol, <i>p</i> -	SS	30.00	46.00	μg/cig	23
Cresol, <i>p</i> -	SS	45.00	62.00	μg/cig	25
Cyclopentenone, 2, 3-dimethyl-2-	MS	9.00	23.00	μg/cig	25
Cyclopentenone, 2, 3-dimethyl-2-	SS	21.00	39.00	μg/cig	25
Cyclopentenone, 2-	MS	21.00	27.00	μg/cig	25
Cyclopentenone, 2-	SS	70.00	103.00	μg/cig	25
Cyclopentenone, 2-OH-3-methyl-2-	MS	3.00	5.00	μg/cig	25
Cyclopentenone, 2-OH-3-methyl-2-	SS	24.00	30.00	μg/cig	25
Cyclopentenone, 2-methyl-2-	MS	17.00	22.00	μg/cig	25
Cyclopentenone, 2-methyl-2-	SS	49.00	95.00	μg/cig	25
Dibenz[<i>a</i> , <i>j</i>]anthracene	IA	6.00		ng/m ³	15
Dibenz[<i>a</i> , <i>j</i>]anthracene	MS	11.00		ng/cig	14
Dibenz[<i>a</i> , <i>j</i>]anthracene	SS	41.00		ng/cig	14
Ethylbenzene (breath, non-smokers)	IA	0.80		μg/m ³	30
Ethylbenzene (breath, smokers)	IA	2.60		μg/m ³	30
Ethylbenzene (homes, non-smokers)	IA	3.50	5.10	μg/m ³	30
Ethylbenzene (homes, smokers)	IA	3.50	8.30	μg/m ³	30
Ethylmethylenepheneanthrene	IA			Qual (ng/m ³)	1
Fluoranthene	IA			Qual (ng/m ³)	1
Fluoranthene	IA	99.00		ng/m ³	15
Fluoranthene	MS	272.00		ng/cig	14
Fluoranthene	MS, P	61.30		ng/cig	16
Fluoranthene	SS	1255.00		ng/cig	14
Fluoranthene	SS, P	669.00		ng/cig	16
Fluoranthene	SS, V	16.90		ng/cig	16
Formaldehyde	IA	1.50	2.10	ppm	2
Formaldehyde	IA	0.10	0.16	ppm	28
Formaldehyde	SS	80.00	110.00	ppm	2
Formic acid	MS	210.00	478.00	μg/cig	23
Formic acid	SS	341.00	665.00	μg/cig	23
Furaldehyde, 2-	MS	15.00	43.00	μg/cig	25
Furaldehyde, 2-	SS	113.00	290.00	μg/cig	25
Furaldehyde, 5-methyl-	MS	6.00	29.00	μg/cig	25
Furaldehyde, 5-methyl-	SS	20.00	127.00	μg/cig	25
Furfuryl alcohol	MS	18.00	65.00	μg/cig	25
Furfuryl alcohol	SS	73.00	283.00	μg/cig	25

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TABLE 1 (continued)

Compound	Sample type	Concentration range		Units	Ref. ^b
		Low	High		
Furoic acid, 2-	MS	44.00	107.00	µg/cig	23
Furoic acid, 2-	SS	25.00	60.00	µg/cig	23
Glutaric acid	MS	10.00	58.00	µg/cig	23
Glutaric acid	SS	6.00	18.00	µg/cig	23
Glycolic acid	MS	37.00	126.00	µg/cig	23
Glycolic acid	SS	35.00	77.00	µg/cig	23
Guaiacol, 4-vinyl-	SS	24.00	32.00	µg/cig	23
Guaiacol, 4-vinyl-	MS	23.00	36.00	µg/cig	23
Guaiacol, 4-vinyl-	MS	16.00	30.00	µg/cig	25
Guaiacol, 4-vinyl-	SS	15.00	37.00	µg/cig	25
HCN	IA	0.01	0.08	mg/m ³	19
HCN (gas only + people)	IA	82.00	86.00	µg/m ³	19
HCN (people absent)	IA	50.00		µg/m ³	19
HCN (people present)	IA	10.00	14.00	µg/m ³	19
Hydrazine	MS	31.50		ng/cig	21
Hydrazine	SS	94.20		ng/cig	21
Hydroquinone	MS	114.00	300.00	µg/cig	23
Hydroquinone	SS	91.00	285.00	µg/cig	23
Hydroquinone, methyl-	MS	23.00	39.00	µg/cig	23
Hydroquinone, methyl-	SS	21.00 ^a	41.00	µg/cig	23
Hydroxypropionic acid, 3-	MS	2.00	31.00	µg/cig	23
Hydroxypropionic acid, 3-	SS	1.00	29.00	µg/cig	23
Indeno[1, 2, 3-cd]pyrene	IA			Qual (ng/m ³)	1
Indeno[1, 2, 3-cd]pyrene	MS, V	0.17		ng/cig	16
Indeno[1, 2, 3-cd]pyrene	SS, P	51.00		ng/cig	16
Indeno[1, 2, 3-cd]pyrene	SS, V	0.36		ng/cig	16
Indeno[1, 2, 3-cd]pyrene	MS, P	8.10		ng/cig	16
Isoquinoline	MS	1.60	2.00	µg/cig	24
Isoquinoline	SS	5.00	8.00	µg/cig	24
Lactic acid	MS	63.00	174.00	µg/cig	23
Lactic acid	SS	45.00	123.00	µg/cig	23
Levulinic acid	MS	29.00	56.00	µg/cig	23
Levulinic acid	SS	25.00	49.00	µg/cig	23
Limonene	MS	15.00	49.00	µg/cig	25
Limonene	SS	63.00	397.00	µg/cig	25
Lutidine, 2, 4-	SS	35.00	315.00	µg/cig	6
Lutidine, 2, 6-	MS	1.40	33.00	µg/cig	6
Lutidine, 2, 6-	SS	1.40	33.00	µg/cig	6
Lutidine, 3, 5-	MS	0.00	17.00	µg/cig	6
Lutidine, 3, 5-	SS	22.00	251.00	µg/cig	6
Methylenephenanthrene, 4, 5-	IA			Qual (ng/m ³)	1
Methylnaphthalene, 1-	SS	30.00		µg/cig	26
Methylnaphthalene, 1-	MS	1.02		µg/cig	26
Methylnaphthalene, 2-	MS	1.21		µg/cig	26
Methylnaphthalene, 2-	SS	31.60		µg/cig	26

TABLE 1 (cont)

Compound
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Methylphenanth
Myosmine
Myosmine
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Nitrosoanabasine
Nitrosoanatinine
Nitrosoanatinine

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TABLE 1 (continued)

Compound	Sample type	Concentration range		Units	Ref. ^b
		Low	High		
Methylnitrosoamino-pyridyl-butanone	MS	46.00	240.00	ng/cig	8
Methylnitrosoamino-pyridyl-butanone	SS	201.00	540.00	ng/cig	8
Methylphenanthrene, 1-	IA			Qual (ng/m ³)	1
Methylphenanthrene, 2-	IA			Qual (ng/m ³)	1
Methylphenanthrene, 3-	IA			Qual (ng/m ³)	1
Methylphenanthrene, 4/9-	IA			Qual (ng/m ³)	1
Myosmine	MS	13.10	33.00	μg/cig	24
Myosmine	SS	73.00	224.00	μg/cig	24
Naphthalene	MS	2.76		μg/cig	26
Naphthalene	SS	45.50		μg/cig	26
Neophytadiene	MS	66.00	232.00	μg/cig	25
Neophytadiene	SS	70.00	421.00	μg/cig	25
Nicotine	IA	25.00	1010.00	μg/m ³	3
Nicotine	IA	0.70	3.10	μg/m ³	17
Nicotine	IA	1.00	10.30	μg/m ³	18
Nicotine	IA	1.70	180.00	pg/m ² min	32
Nicotine	MS	1720.00	3330.00	μg/cig	24
Nicotine	MS	1483.00	3149.00	μg/cig	25
Nicotine	SS	3210.00	5830.00	μg/cig	24
Nicotine	SS	2987.00	6588.00	μg/cig	25
Nicotine (gas only + people)	IA			Traces only	19
Nicotine (people absent)	IA	130.00		μg/m ³	19
Nicotine (people present)	IA	102.00		μg/m ³	19
Nicotine, office buildings	IA	1.70	180.00	pg/m ² min	32
Nicotyrine	MS	4.20	20.20	μg/cig	24
Nicotyrine	MS	17.00	41.00	μg/cig	25
Nicotyrine	SS	49.00	211.00	μg/cig	24
Nicotyrine	SS	93.00	263.00	μg/cig	25
Nitrogen dioxide	IA	0.00	0.03	ppm	19
Nitrogen dioxide	IA	58.00		ppb	31
Nitrogen dioxide (gas only + people)	IA	0.01	0.03	ppm	19
Nitrogen dioxide (people absent)	IA	0.00		ppm	19
Nitrogen dioxide (people present)	IA	0.00		ppm	19
Nitrogen dioxide control	IA	27.00		ppb	31
Nitrogen oxide	IA	0.30	0.60	ppm	19
Nitrogen oxide	IA	0.00	9.00	ppb	31
Nitrogen oxide (gas only + people)	IA	0.31	0.40	ppm	19
Nitrogen oxide (people absent)	IA	0.48	0.59	ppm	19
Nitrogen oxide (people present)	IA	0.30	0.60	ppm	19
Nitrogen oxide control	IA	5.00		ppb	31
Nitrogen oxides (combined)	IA	59.00	218.00	ppb	12
Nitrosoamine, methylethyl-	MS	0.10	9.10	ng/cig	5
Nitrosoamine, methylethyl-	MS	0.00	1.80	ng/cig	7
Nitrosoamine, methylethyl-	SS	9.00	75.00	ng/cig	5
Nitrosoamine, methylethyl-	SS	0.00	27.00	ng/cig	7
Nitrosoanabasine, N'-	SS	15.00	40.00	ng/cig	8
Nitrosoanatinidine, N'-	MS	82.00	167.00	ng/cig	8
Nitrosoanatinidine, N'-	SS	61.00	220.00	ng/cig	8

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EXHIBIT 1 (continued)

[illegible]

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TABLE 1 (continued)

Compound	Sample type	Concentration range		Units	Ref. ^b
		Low	High		
Phenol, 4-vinyl-	MS	18.00	45.00	μg/cig	25
Phenol, 4-vinyl-	SS	25.00	57.00	μg/cig	25
Phenols	IA	7.40	11.50	μg/m ²	20
Phenylacetic acid	MS	18.00	38.00	μg/cig	23
Phenylacetic acid	SS	11.00	30.00	μg/cig	23
Picoline, 3-	MS	12.00	22.00	μg/cig	25
Picoline, 3-	SS	90.00	166.00	μg/cig	25
Picoline, alpha-	MS	12.30	189.00	μg/cig	6
Picoline, alpha-	SS	128.00	1090.00	μg/cig	6
Pyran-4-one, 5, 6-diOH-3, 5-diOH-2-ME	MS	13.00	153.00	μg/cig	25
Pyran-4-one, 5, 6-diOH-3, 5-diOH-2-ME	SS	0.00	143.00	μg/cig	25
Pyrazine, 2, 3-dimethyl-	SS	0.00	50.00	μg/cig	6
Pyrazine, 2-methyl	MS	0.00	8.60	μg/cig	6
Pyrazine, 2-methyl-	SS	0.00	8.60	μg/cig	6
Pyrene	IA			Qual (ng/m ³)	1
Pyrene	IA	66.00		ng/m ³	15
Pyrene	IA	4.10	9.40	ng/m ³	20
Pyrene	MS	270.00		ng/cig	14
Pyrene	MS, P	43.00		ng/cig	16
Pyrene	MS, V ^a	1.90		ng/cig	16
Pyrene	SS	1011.00		ng/cig	14
Pyrene	SS, P	466.00		ng/cig	16
Pyrene	SS, V	10.30		ng/cig	16
Pyrene control	IA	2.80	7.00	ng/m ³	20
Pyrene, 1-methyl-	IA			Qual (ng/m ³)	1
Pyrene, 2-methyl-	IA			Qual (ng/m ³)	1
Pyrene, 4-methyl-	IA			Qual (ng/m ³)	1
Pyridine	MS	32.40	648.00	μg/cig	6
Pyridine	MS	16.00	20.00	μg/cig	25
Pyridine	SS	336.00	3420.00	μg/cig	6
Pyridine	SS	187.00	262.00	μg/cig	25
Pyridine, 2-(3-pentyl)-	MS	0.00	1.50	μg/cig	6
Pyridine, 2-(3-pentyl)-	SS	0.00	143.00	μg/cig	6
Pyridine, 2-ethyl-	MS	2.60	35.00	μg/cig	6
Pyridine, 2-ethyl-	SS	2.60	35.00	μg/cig	6
Pyridine, 3-acetyl-	MS	3.80	6.40	μg/cig	24
Pyridine, 3-acetyl-	SS	9.00	11.00	μg/cig	24
Pyridine, 3-cyano-	SS	24.00	64.00	μg/cig	24
Pyridine, 3-cyano-	MS	2.40	4.20	μg/cig	24
Pyridine, 3-ethyl-	MS	4.00	6.00	μg/cig	25
Pyridine, 3-ethyl-	SS	71.00	960.00	μg/cig	6
Pyridine, 3-ethyl-	SS	21.00	36.00	μg/cig	25
Pyridine, 3-ethyl-4-methyl-	SS	6.40	34.00	μg/cig	6
Pyridine, 3-ethyl-4-methyl-	MS	0.00	1.50	μg/cig	6
Pyridine, 3-hydroxy-	MS	125.10	211.40	μg/cig	24
Pyridine, 3-hydroxy-	MS	90.00	119.00	μg/cig	25
Pyridine, 3-hydroxy-	SS	152.00	167.00	μg/cig	24
Pyridine, 3-hydroxy-	SS	157.00	191.00	μg/cig	25
Pyridine, 4-ethyl-	SS	27.00	379.00	μg/cig	6
Pyridine, 4- <i>i</i> -butyl-	MS	0.00	4.50	μg/cig	6
Pyridine, 4- <i>i</i> -butyl-	SS	17.00	287.00	μg/cig	26
Pyridine, methylvinyl-	MS	2.20	4.10	μg/cig	24
Pyridine, methylvinyl-	SS	12.00	19.00	μg/cig	23

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TABLE 1 (continued)

Compound	Sample type	Concentration range		Units	Ref. ^b
		Low	High		
Pyrrole	MS	16.00	23.00	μg/cig	25
Pyrrole	SS	140.00	272.00	μg/cig	25
Styrene (breath, non-smokers)	IA	0.30		μg/m ³	30
Styrene (breath, smokers)	IA	1.10		μg/m ³	30
Styrene (homes, non-smokers)	IA	0.80	1.10	μg/m ³	30
Styrene (homes, smokers)	IA	1.10	2.20	μg/m ³	30
Succinic acid	MS	112.00	163.00	μg/cig	23
Succinic acid	SS	65.00	70.00	μg/cig	23
Succinic acid, methyl-	MS	4.00	31.00	μg/cig	23
Succinic acid, methyl-	SS	1.00	13.00	μg/cig	23
Tar radical sol in <i>n</i> -butylbenzene	MS			Qualitative	22
Tar radical sol in <i>n</i> -butylbenzene	SS			Qualitative	22
Thiocyanate (plasma, non-smoker)	IA	70.80		μmol/l	28
Thiocyanate (plasma, passive smoker)	IA	71.80		μmol/l	28
Thiocyanate (plasma, smoker)	IA	70.70		μmol/l	28
Thioethers (urine, non-smoker)	IA	6.00		mmol/ml	28
Thioethers (urine, passive)	IA	6.40		mmol/ml	28
Thioethers (urine, smoker)	IA	6.30		mmol/ml	28
Toluene	IA	0.04	1.04	mg/m ³	30
Valeric acid, 3-methyl-	MS	20.00	261.00	μg/cig	23
Valeric acid, 3-methyl-	SS	20.00	384.00	μg/cig	23
Vinylphenol, <i>p</i> -	MS	21.00	51.00	μg/cig	23
Vinylphenol, <i>p</i> -	SS	21.00	45.00	μg/cig	23
Xylene, <i>m</i> + <i>p</i> - (breath, non-smokers)	IA	2.10		μg/m ³	30
Xylene, <i>m</i> + <i>p</i> - (breath, smokers)	IA	5.50		μg/m ³	30
Xylene, <i>o</i> - (breath, non-smokers)	IA	0.80		μg/m ³	30
Xylene, <i>m</i> + <i>p</i> - (homes, non-smokers)	IA	10.00	13.00	μg/m ³	30
Xylene, <i>m</i> + <i>p</i> - (homes, smokers)	IA	10.00	20.00	μg/m ³	30
Xylene, <i>o</i> - (breath, smokers)	IA	1.60		μg/m ³	30
Xylene, <i>o</i> - (homes, non-smokers)	IA	4.00	5.20	μg/m ³	30
Xylene, <i>o</i> - (homes, smokers)	IA	3.20	7.10	μg/m ³	30
Xylenol, 2, 6-	MS	8.00	16.00	μg/cig	23
Xylenol, 2, 6-	SS	8.00	20.00	μg/cig	23

^a Listings are given in alphabetical order by compound and special conditions are noted in parentheses within the compound name. The sample type is categorized in the second column as IA, indoor air; SS, sidestream smoke; MS, mainstream smoke; P, associated with particulate matter; and V, associated with volatile compounds.

^b Reference numbers are as follows: 1, Alfheim and Ramdahl, 1984; 2, Ayer and Yeager, 1982; 3, Badre et al., 1978; 4, Brunne-
mann et al., 1976; 5, Brunne-
mann et al., 1977; 6, Brunne-
mann et al., 1978; 7, Brunne-
mann et al., 1980; 8, Brunne-
mann and Hoffmann, 1975; 9, Brunne-
mann and Hoffmann, 1978; 10, Elliott and Rowe, 1975; 11, Fischer et al.,
Galuskinova, 1964; 12, Grimmer et al., 1977a; 13, Grimmer et al., 1977b; 14, Grimmer et al., 1987; 15, Harmsen and
1957; 16, Hinds and First, 1975; 17, Hugod et al., 1978; 18, Just et al., 1972; 19, Liu et al., 1974; 20, Pryor et al., 1983; 21, Sakuma et al., 1984a; 22, Sakuma et al., 1984b; 23, Schmeltz et al., 1976; 24, Sexton et al., 1984; 25, Siehlik et al., 1982; 26, Wallace and Pellizzari, 1986; and 27, Williams et al., 1985.

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genotoxicity, one may calculate, in an additive fashion, the 'total genotoxic potential' of a cigarette. Comparative human exposure could then be calculated by knowing the total genotoxic potential of a cigarette, room air volume, and the air exchange rate. If one were to make a crude estimate of the bacterial mutagenicity of 'a cigarette' by using the upper concentration ranges from Table 1 and *Salmonella* potency range for the 10 bacterial mutagens (1 rev/100 mg to 1 rev/0.10 μ g), the calculated mutagenic potency [μ g of compound/cigarette \times revertants/ μ g compound] of a cigarette would be approximately 0.5–45 revertants per cigarette. If all of the compounds in Table 2 except the known negatives were to be shown to be *Salmonella* mutagens, one could estimate the mutagenic activity level to be as high as 1500 revertants/cigarette. Obviously, this method of summing the total genotoxic activity for a 'typical cigarette' is most useful when the chemical and bioassay values are quantitatively accurate; when all genotoxicants have been identified and bioassayed properly, and when synergistic and/or antagonistic interactions do not interfere. Because one would not expect this list of bacterial mutagens to be inclusive of all the mutagens and because one cannot presently rule out all possible types of interactions, this type of calculation would estimate only the lower limits of mutagenicity as will be clearly demonstrated below.

Upon testing an acetone extract of SS particles, Löfroth et al. (1983) found the extract preferentially mutagenic in *S. typhimurium* TA98 in the presence of S9. The observed response using TA98 corresponded to 15 000 revertants per cigarette. Upon using a cigarette-smoking machine in a 15-m³ room, Löfroth and Lazaridis (1986) calculated that their results represent the equivalent of 30 000 revertants per cigarette for MS and 10 000–20 000 revertants per cigarette for SS when using *S. typhimurium* TA98 with S9 activation. In 1987, Ling et al., using the same methods, observed for strain TA98 a range of 17 200–31 300 revertants per cigarette for SS organics tested using the plate incorporation protocol and a range of 36 000–118 300 revertants per cigarette using a micro-assay preincubation protocol. In addition, they observed significant activity using TA100 without

TABLE 2

THE GENOTOXICITY OF COMPOUNDS ASSOCIATED WITH ENVIRONMENTAL TOBACCO SMOKE

Compound	CAS Number	Bioassay results ^a	
Acetamide	60-35-5	CCC	+
		CT	+
		ST	NEG
Acetic acid	64-19-7	ST	NEG
Acrolein	107-02-8	ST	+
Anthracene	120-12-7	CCC	I
		CT	NEG
		CYC	NEG
		MNT	NEG
		ST	NEG
Benz[<i>a</i>]anthracene	56-55-3	CCC	+
		CT	+
		REC	NEG
		ST	+
		V79	+
Benzene	71-43-2	CCC	+
		CYI	+
		MNT	+
		SCE	NEG
		TRM	+
Benzo[<i>a</i>]pyrene	50-32-8	CCC	+
		CT	+
		CYC	NEG
		MDR	+
		MNT	+
		MNT	+
		MST	+
		SCE	+
		SRL	+
		ST	+
Benzo[<i>b</i>]fluoranthene	205-99-2	CCC	+
		CT	+
Benzo[<i>e</i>]pyrene	192-97-2	CCC	I
		CT	NEG
		REC	NEG
Benzo[<i>ghi</i>]perylene	191-24-2	ST	+
Benzoic acid	65-85-0	ST	NEG
Butyrolactone, gamma	96-48-0	ST	NEG
Cresol, <i>m</i> -, <i>o</i> -, and <i>p</i> -	95-48-7	ST	NEG
Dibenz[<i>a,j</i>]anthracene	224-41-9	ST	+
Formaldehyde	50-00-0	ASPD	+
		CCC	+
		MDR	+
		NEU	+
		REC	+
		SRL	+
Hydrazine	302-01-2	SRL	+
		ST	+
		CCC	+
		ST	+

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TABLE 2 (continued)

Compound	CAS Number	Bioassay results ^a	
Hydroquinone	123-31-9	ALC	+
		ST	NEG
Indeno[1, 2, 3- <i>cd</i>]pyrene	193-39-5	CCC	+
		ST	NEG
Isoquinoline	119-65-3	ST	NEG
Limonene	5989-27-5	ST	NEG
Naphthalene	91-20-3	ST	NEG
Nicotine	54-11-5	NEU	NEG
		ST	NEG
Nitrosodiethylamine, <i>N'</i> -	55-18-5	CCC	+
		CT	+
		CYC	+
		L5	+
		MDR	+
		MST	NEG
		REC	+
		SCE	+
		ST	+
		V79	+
		ARA	+
		CCC	+
		CT	+
Nitrosodimethylamine, <i>N'</i> -	62-75-9	CYG	+
		CYG	NEG
		L5	+
		MNT	+/-
		MST	NEG
		NEU	+
		SCE	+
		SRL	+
		ST	+
		V79	+
Nitrosonornicotine	16543-55-8	YEA	+
Nitrosopyrrolidine	930-55-2	CCC	+
		CCC	+
Perylene	198-55-0	SCE	NEG
		ALC	+
Phenanthrene	85-01-8	CCC	I
		CT	NEG
		CYC	NEG
		ST	NEG
Phenol	108-95-2	NEU	NEG
		ST	NEG
Pyrene	129-00-0	CCC	I
		CT	NEG
		CYC	NEG
		ST	NEG
		V79	NEG
Pyridine	110-86-1	SCE	+
		ST	NEG

TABLE 2 (continued)

Compound	CAS Number	Bioassay results ^a	
Styrene	100-42-5	CCC	-
Toluene	108-88-3	SCE	NEG
		ST	NEG

^a Bioassay information is extracted from Graedel et al. (1984). Abbreviations used for bioassay results are as follows: ALC, Allium cytogenetics assays; ARA, Arabidopsis mutagen assay; ASPH, Aspergillus mutagen assay; CCC, whole animal carcinogen assays; CT, cell transformation bioassays; CY, mammalian cytogenetic bioassays; L5, L5178Y mouse lymphoma assay; MDR, mammalian cell DNA repair assays; MNT, micronucleus assays; MST, mouse spot test; NEU, Neurospora assays; REC, DNA repair-deficient bacterial assays; SCE, sister-chromatid exchange assay; SRL, sex-linked recessive lethal assays in *Drosophila*; SI, *Salmonella* assays; TRM, *Tradescantia* mutagen assays; V79, V79 Chinese hamster mutation assays; and YEA, Yea mutation tests. Results are recorded as +, positive; -, negative; and I, Indefinite.

activation. Ong et al. (1984) examined the mutagenicity of SS using *S. typhimurium* TA98 (an antibiotic-resistant strain of TA98) in a plate impinger system. They stated that "the concentration of cigarette smoke that could be detected for mutagenic activity was 0.0065 cigarettes per ml." Because the concentration of cigarette smoke in these experiments was equivalent to approximately 0.036 cigarettes/ml and the observed response was approximately 4 revertants per [(40 rev/ml) · (1 ml/0.036 cig)] revertants per cigarette. However, if one assumes that the calculations that 0.0065 cigarettes would cause a doubling (~20 induced revertants) of the spontaneous revertant number, the activity of SS is approximately 3080 [(20 rev/doubling) · (doubling/0.0065 cigarettes)] revertants per cigarette. The 3- to 10-fold difference between the results of the Löfroth et al. (1983) and Ong et al. (1984) is most likely due to differences in the collection and exposure systems used, although, once again, one cannot rule out the role of differences in chemical interactions. Löfroth et al. (1983) collected only particulate matter and tested the extracted organic material, whereas Ong et al. (1984), while attempting to test total SS, probably assayed primarily the

in particulate, so would dissolve in methods of Löfroth (1983) did not identify responsible for the pathway of the accurate determination of a cigarette.

Knowing the constituents of a typical cigarette case that one can put to genotoxic and SS, the amount emitted from a gram per cigarette levels. The cigarette-imparted (mg/m³); however, concentrations from assays simple by smoking rate, volume of the cigarette. Also, each emitted from the exposure levels. The respirable portion of the level of with the particulate residence can vary approximately 1000 times. The research Council. concentrations (Table) at least 2 orders of magnitude amounts of different from the samples. It is also have been identified to matter organic matter. The modified bioassay indicated that the cigarette volume of cigarette smoking cigarettes smoking, however, due to the mutagens in

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nonparticulate, semi-volatile organic material that would dissolve in the fluid medium. Although the methods of Löfroth et al. (1983) and Ong et al. (1984) did not identify the specific genotoxins responsible for the biological activity, the direct bioassay of the emissions did provide a more accurate determination of the total mutagenic potential of a cigarette.

Knowing the amounts and bioassay activity of SS constituents or the total mutagenic activity of a 'typical cigarette' also does not necessarily indicate that one can accurately estimate human exposure to genotoxic (ETS) emissions. For both MS and SS, the amount of a specific material that is emitted from a cigarette ranges from sub-nanogram per cigarette levels to milligram per cigarette levels. The concentrations of constituents in smoke-impacted spaces range from ng/m³ to mg/m³; however, the calculation of ambient concentrations from cigarette emission rates is not always simple because ETS levels are functions of smoking rate, ventilation, sink rate, mixing, and volume of the space (National Research Council, 1986). Also, each individual compound may be removed from the ambient air at different rates due to these functions. For example, genotoxicant exposure levels can be approximated by measuring respirable particulate (RSP) levels and measuring the level of specific genotoxins associated with the particles. RSP levels in a one-smoker residence can vary by 3 orders of magnitude from approximately 17 to 5000 µg/m³ (National Research Council, 1986). Similarly, most IA concentrations (Table 1) for specific compounds span at least 2 orders of magnitude; however, the relative amounts of components with SS are often different from the relative amounts found in IA samples. It is also unlikely that all SS carcinogens have been identified. By collecting indoor air particulate matter and bioassaying the extracted organic matter using *S. typhimurium* TM677 in modified bioassay, Lewtas et al. (1987) demonstrated that particle-associated mutagenicity per cubic volume of air was greatest in the homes with cigarette smoking and correlated with the number of cigarettes smoked. The mutagenic activity per cigarette, however, could not be calculated in this study due to the presence of other potential sources of mutagens such as woodstoves, gas appliances,

cooking, etc. Recently, Husgafvel-Pursiainen et al. (1986) demonstrated that indoor airborne particulate matter collected in 3 restaurants where smoking occurred gave up to 2370 revertants/m³ air. On the basis of optical particle counting, they attributed the majority of the airborne particulate matter to cigarette smoking; however, they also did not estimate mutagenicity on a per cigarette basis. Husgafvel-Pursiainen et al. (1986), however, did show that the levels of polynuclear aromatic compounds roughly correlated with the mutagenic activity. Human exposure to tobacco-smoke genotoxins, therefore, is highly variable and difficult to assess by evaluating individual components and/or equivalent bioassay activity levels emitted into ambient air.

In order to explore the use of bacterial bioassays in evaluating ETS, our laboratory has examined some of the alternative methods that can be used for evaluating ETS. The approaches used are briefly described in the Materials and methods section. As part of one study (Morin et al., 1987), filters used to collect SS particles were extracted with various solvents, solvent-exchanged to DMSO, and bioassayed. Depending upon the extraction conditions, tester strain, and activation conditions the revertants per cigarette ranged from 400 to 19000 (Fig. 1). Results using TA98 with S9 of ~19000 revertants/cigarette resemble data obtained by Löfroth et al. (1983).

Because the work of Ong et al. (1984) demonstrated that the semi-volatile and volatile components of SS may be mutagenic, we decided to assess the total mutagenic potential of both MS and SS. Using the sequential trapping train method described, we collected volatile, semi-volatile, and particulate-bound organics simultaneously from both MS and SS using separate trapping trains. Each of the fractions from both trains was bioassayed separately in the *Salmonella* mutagenicity assay. No mutagenicity was associated with the cold trap (volatile fraction) of either the MS or SS when evaluated as gases in the Tedlar dessicator system (Hughes et al., 1987). Table 3 summarizes the results for the other components of the trapping trains. Both the bubbler and sand trap samples were mutagenic for MS and SS; therefore, both the particulate and the semi-volatile components were mutagenic. The revertants/cigarette

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TEST CONDITION: (Solvent/Strain/Activation)

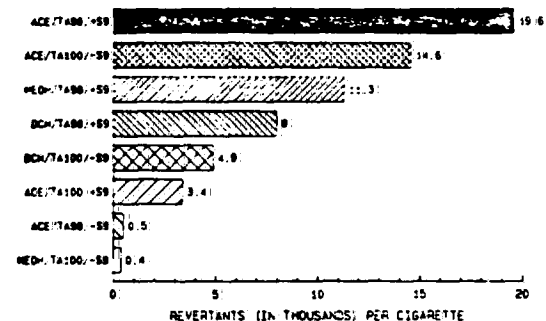


Fig. 1. *Salmonella typhimurium* mutagenicity of sidestream cigarette smoke when tested using various solvent extraction systems; strains of *S. typhimurium*; and activation conditions. Abbreviations are as follows: ACE, Acetone; DCM, Dichloromethane; MEOH, Methanol; TA98 and TA100 for respective strains of *S. typhimurium*; +S9, with exogenous activation; and -S9 without exogenous activation.

value of 35 200 is approximately twice the 19 000 and 15 000 values previously seen with particle-associated fractions; however, when the cigarette machine was not used to generate a puff mode sample and the cigarettes were allowed to smolder, this value was reduced to 23 250 revertants per cigarette. It has been demonstrated previously that the products produced during puffing and natural smoldering are somewhat different due to generated temperatures, the rate of mass transfer of oxygen to the tobacco source, properties of the cigarette paper, etc. (Baker, 1981, 1982). Therefore, it is reasonable that the mutagenicity of both MS and SS varies with the degree of puffing. The

effect of burn conditions can also be noted by examining the revertants/ μg of organic material values in Table 3. Compared on a per mass of organic material basis, the MS organics are 2-3 times as mutagenic as the SS organics. This demonstrates that the mainstream and sidestream combustion processes produce different relative amounts of bacterial mutagens. One also could speculate - from knowledge of the 2 types of combustion processes and differences in MS and SS chemistry - that the quantitative distribution of mutagens in MS and SS is different. Due to the mass of organic material produced, however, SS organics make a larger contribution to the total mutagenic activity of a cigarette. It is interesting to note that within our laboratory the response of benzo[*a*]pyrene (B[a]P) in *Salmonella typhimurium* TA98 with exogenous activation is approximately 20 revertants/ μg ; therefore, one can calculate that total cigarette bacterial mutagenicity is equivalent to ~ 2.6 mg of B[a]P. Approximately 7% (61 600/86 300) of the total mutagenicity is associated with the sand trap (particulate) sample. This proportion also is approximately the same in the MS and SS samples. Overall, the SS sample accounted for approximately 60% of the total mutagenicity on a per cigarette basis.

The best way presently available to assess human exposure to genotoxic ETS compounds is the analysis of the fluids and tissues of exposed individuals. Exposure of target tissues and subsequent genotoxic constituents of ETS depends on several factors, including the number of cigarettes smoked

in an enclosed area, the degradation rates and volume of air in the body distribution. The uptake of carcinogens is determined by the rate at which one can measure carboxyhemoglobin levels in the biological fluid. Thiocyanate degradation of hemoglobin from exclusively from 1986). The quantitative and biochemical hydroxyproline amines are still Research Council bioassays are known whether compounds are absorbed or excreted at the site of action or in a noncompartmental model. In genotoxicity, the advantage of the Ames test for mutagenicity provides a *in vivo* exposure assessment. Yamasaki and co-workers (1983) found bacterial mutagenicity by IAR confirmed the results, however, there were discrepancies in these studies. In dietary factors that have examined in smokers (1983; Sorsa et al., 1983) mutagenicity is not generally observed and did not observe the results might be. In spite of the support the use of the Ames test tool for mutagenicity; however, puffing and standing, the proper factors such as In summary,

TABLE 3

THE *SALMONELLA TYPHIMURIUM* TA98 MUTAGENICITY OF MAINSTREAM AND SIDESTREAM TOBACCO SMOKE COLLECTED IN A PUFF MODE

Fraction	Ethanol bubbler		Sand trap		Bubbler + sand
	Rev/ μg ^a	Rev/cig ^b	Rev/ μg	Rev/cig	Rev/cig
Main	8.1	7557	4.0	26 400	33 957
Side	2.7	17 099	1.6	35 200 ^c	52 299
Total		24 656		61 600	86 256

^a *S. typhimurium* TA98 mean revertants per plate per μg of organic material collected using a preincubation bioassay. Data were analyzed using a linear regression model (Myers et al., 1981).

^b *S. typhimurium* TA98 revertants per cigarette calculated from (Rev/ μg) \times (μg of fraction/cigarette).

^c When puff mode was not used and the cigarettes were allowed to smolder, this value was 23 253 rev/cig.

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in an enclosed area, the size and nature of the area, the degree of room ventilation, breathing rates and volume, absorption of the genotoxics, body distribution and excretion, and metabolism. The uptake of individual agents from ETS can be determined using chemical methods. For example, one can measure products such as thiocyanate, carboxyhemoglobin, nicotine and cotinine in physiological fluids. However, some of these (e.g., thiocyanate due to HCN exposure and carboxyhemoglobin from CO exposure) do not originate exclusively from SS (National Research Council, 1986). The quantitative aspects of other chemical and biochemical markers such as cotinine, hydroxyproline, *N*-nitrosoproline, and aromatic amines are still somewhat questionable (National Research Council, 1986). In addition, when one bioassays an agent individually, it may not be known whether or not different genotoxic compounds are absorbed, distributed, metabolized, and excreted at the same rate as each other. The use of a noncompound-specific measurement of exposure to genotoxics, therefore, would be very advantageous. The monitoring of nonsmokers' urine for mutagenic potential using bacterial assays provides a possible means of evaluating in vivo exposure to ETS genotoxics. In 1977, Yamasaki and Ames reported the presence of bacterial mutagens in the urine of smokers. As noted by IARC (1986), a number of studies have confirmed the finding of Yamasaki and Ames; however, there is a wide variation in the results of these studies. Much of this variation may be due to dietary factors (Sasson et al., 1985). The studies that have examined the urine mutagenicity of passive smokers (Putzrath et al., 1981; Bos et al., 1983; Sorsa et al., 1985) demonstrated increased mutagenicity in adult passive smokers. These studies generally examined small numbers of people and did not control for dietary factors; therefore, the results might be considered somewhat ambiguous. In spite of the shortcomings, these studies did support the use of bacterial bioassays as a screening tool for human exposure to ETS genotoxics; however, more effort is needed in improving and standardizing the methods and in creating the proper controls for other environmental factors such as diet.

In summary, our studies support previous stud-

ies that demonstrated that ETS particle-bound organic material is mutagenic. In addition, our studies demonstrated that some semi-volatile and volatile components were mutagenic; however, the highly volatile compounds (for both MS and SS) collected in the third-stage liquid nitrogen cold trap were not mutagenic. Within these studies, the total mutagenicity was divided among 4 fractions approximately as follows: SS sand trap (particulate) fraction, 40%; MS sand trap (particulate) fraction, 30%; SS solvent (semi-volatile) fraction, 20%; and MS solvent (semi-volatile) trap fraction, 10%. Results also gave an indication that the frequency with which a cigarette is puffed affects the total amount of mutagenic material produced. Although these studies illustrate the usefulness of bacterial mutagenicity bioassays for characterizing ETS, there are also other uses (e.g., identifying specific genotoxics) for which bacterial assays will find great utility.

Acknowledgements

The technical assistance of Linda Monteith, Debra Simmons, Jack Callahan, Ken Davis, and Jeff Keever is gratefully appreciated. The authors wish to thank Dr. Göran Löfroth and Ms Virginia Houk for their careful review and suggestions for the manuscript. The research described in this paper has been reviewed by the Health Effects Research Laboratory, U.S. Environmental Protection Agency, and approved for publication. Approval does not signify that the contents necessarily reflect the views and policies of the Agency nor does the mention of trade names or commercial products constitute endorsement or recommendation for use.

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Public exposure to environmental tobacco smoke

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(Received 25 November 1987)

(Revision received 28 March 1988)

(Accepted 29 March 1988)

Keywords: Environmental tobacco smoke (ETS); Passive smoking; Personal sampling; Salmonella mutagenicity assay; Smoking.

Summary

Airborne particulate matter has been collected by personal samplers in public indoor areas and travel situations with environmental tobacco smoke pollution. Following extraction, the samples were assayed for mutagenicity in the presence of S9 with a sensitive microsuspension test using *Salmonella* TA98. The mutagenic responses of indoor air from public areas were much higher than those of ambient outdoor air. Depending on the circumstances, the mutagenic response varied in trains and airplanes but the results show that physical separation of non-smoking sections from smoking sections is necessary in order to achieve genuine non-smoking areas. Chemical fractionation and mutagenicity assay of the basic fraction show that *Salmonella* mutagenicity of airborne particulate matter might be used as a tobacco smoke-specific indicator, as the basic fraction of environmental tobacco smoke contains a large part of the mutagenic activity whereas this is not the case for outdoor ambient airborne particulate matter.

Environmental tobacco smoke (ETS) is a complex mixture of gases and particulate tar matter comprising numerous compounds. ETS is one of the most common air pollutants in industrialized and urban societies as 25-40% of the adult population are smokers and much smoking takes place indoors causing pollution of the air breathed by everyone.

Among several types of pollution indices, *Salmonella* mutagenicity of airborne particulate matter has been used to study the contribution from ETS in office buildings (Löfroth et al., 1983), restaurants (Husgafvel-Pursiainen et al., 1986) and

homes (van Houdt et al., 1984; Alfheim and Ramdahl, 1984; Löfroth and Lazaridis, 1986; Lewtas et al., 1987). In some studies (Löfroth et al., 1983; Löfroth and Lazaridis, 1986) the origin of the mutagenic activity was ascertained by fractionation in which a major part of the activity of ETS was recovered in the basic fraction.

The use of personal samplers for the collection of particulate matter coupled with a more sensitive mutagenicity test, a *Salmonella* microsuspension assay, was recently explored and found feasible (Ling et al., 1987). These studies have now been extended with measurements of the mutagenic response of airborne particulate matter collected during some typical situations outside home and work where involuntary exposure to ETS can occur. The chemical behavior of ETS as compared

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to outdoor ambient particulate matter with respect to the contribution from the basic fraction has also been further studied.

Materials and methods

Sampling

Indoor airborne particulate matter was collected on glass fiber filters with 2 battery-operated personal samplers (Casella AFC 123; Casella London Inc., Great Britain) using a flow rate of 2 l/min for each sampler and 25-mm sampling heads. The samplers were carried in a small bag keeping the sampling heads near the breathing zone, i.e. no more than about 25 cm below the mouth/nose region. Sampling time varied from less than 1 h to more than 6 h depending on location. The filters were stored in Al-foil at -20°C within 2 h of the end of the sampling.

Parallel sampling of outdoor airborne particulate matter was performed with a battery-operated portable sampler (Casella HFS 800) using a flow rate of 10 l/min and a 35-mm sampling head. The sampling was made from a car parked at the nearby parking lot with the air intake of the sampler placed outside the car.

Experimental sidestream smoke was collected as described previously (Ling et al., 1987) from machine-smoked cigarettes in a small 0.3-m³ hood. A high-volume sample of glass fiber filter-collected urban airborne particulate matter was also used for comparison.

Sample preparation

The filters were extracted within 10 days after sampling. Extraction was performed with acetone using a bath-type sonicator. The solution was filtered through a No. 4 glass filter to remove glass fiber debris.

Fractionation with respect to polarity into basic and non-basic fractions was performed by liquid-liquid extraction with diethyl ether and sulfuric acid and sodium hydroxide aqueous solutions as described earlier (Löfroth, 1981).

For the microsuspension assay, aliquots of the extract corresponding to known air volumes were transferred to 11 mm \times 75 mm sterile glass tubes containing 5 μ l dimethyl sulfoxide (DMSO). The acetone was evaporated by a stream of nitrogen

gas with the tubes inserted in a heating block and the evaporating solution was kept at $< 40^{\circ}\text{C}$. The tubes were finally stoppered using sterile silicon stoppers and stored at -20°C until they were used for the bioassay.

For the plate incorporation assay, the extract was evaporated to a small volume and then diluted with DMSO. The samples were stored at -20°C .

Bioassay

The microsuspension technique (Kado et al., 1983) was used with minor modifications for all environmental samples in the present study. *Salmonella* TA98 grown for about 13 h with rapid shaking was centrifuged and then resuspended in 1/10 of the original volume in Vogel and Bonner (1956) medium E (instead of phosphate-buffered saline) giving approximately 10^{10} cells/ml of which 0.1 ml was added to the sample tubes.

The S9 was obtained from livers of Aroclor 1254-induced male Sprague-Dawley rats. Its protein content was 33 mg/ml as determined by the method of Lowry et al. (1951). The S9 mix was prepared as described by Ames et al. (1975) but with Vogel and Bonner medium E instead of 0.2 M phosphate buffer for the microsuspension assay. Each sample tube received 0.1 ml S9 mix containing 10% S9 or buffer without S9, NADP and glucose 6-phosphate.

The sample tubes, covered with sterile caps, were then immediately incubated for 90 min at 37°C with about 175 rpm shaking, after which 2 ml top agar containing histidine and biotin were added. Following Vortex mixing, the samples were poured on minimal glucose agar plates. The plates were incubated for 48 h at 37°C . Revertant colonies were then counted manually.

The plate incorporation assay was performed as described by Maron and Ames (1983). The S9 mix contained 4 or 10% S9 and 0.5 ml was added to each plate. The S9 amounts employed were those which are routinely used for other outdoor ambient and indoor tobacco smoke samples.

Depending on the amount of sample available, each sample was tested repeatedly on several occasions with 1 plate per dose and 3–5 plates for the spontaneous control. Some samples were only available for 1 or 2 independent tests but larger

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samples were always tested 3 or 4 times. Each test comprised positive control compounds. Blank filter samples have been assayed and not found to give any detectable mutagenic activity.

The dose response was evaluated with least square linear regression using all plate counts in the linear or approximately linear part of the dose-response curve.

Results

Environmental samples

During this study a number of samples of airborne particulate matter have been collected at locations or travel situations which people may experience in their daily life.

Table 1 relates the results from 2 visits to a shopping center in the northern part of the Stockholm area and 2 visits to the Stockholm Central (railway) station. The shopping center is about 12 m high and has a central 25 m \times 45 m indoor plaza with four 45-65-m-long extending alleys. The Central station main hall has an indoor area of 28 m \times 119 m and a height of about 15 m. Despite these spacious designs, the indoor pollution, measured as mutagenic response of particulate matter, is high and is higher than the response of simultaneously collected ambient outdoor air.

Samples collected during train travel (Table 2) were obtained in the common type of passenger cars containing 2 compartments separated by a

TABLE 1
MUTAGENIC ACTIVITY AND RESPONSE IN TA98-S9 OF AIRBORNE PARTICULATE MATTER COLLECTED IN INDOOR PUBLIC AREAS AND SIMULTANEOUSLY AT NEARBY OUTDOOR LOCATIONS

Sample location, date and duration	Indoors			Outdoors		
	Dose (l air/plate)	Counts (rev./plate)	Response (rev./m ³)	Dose (l air/plate)	Counts (rev./plate)	Response (rev./m ³)
Shopping center	0	49		0	49	
861222	25	62		75	46	
175 min	50	112	1200	150	58	< 50
	100	177		300	55	
	150	231				
Benzo(a)pyrene *	0.5 μ g	301				
Shopping center	0	51		0	51	
861230	25	52		75	58	
175 min	50	94	1000	150	82	140
	100	165		300	85	
	150	192				
Benzo(a)pyrene	0.5 μ g	345				
Central station	0	53		0	53	
870116	25	101		200	71	
235 min	50	163	2500	400	149	260
	100	330		600	236	
	200	552				
Benzo(a)pyrene	0.5 μ g	445				
Central station	0	62		0	62	
870130	25	107		200	59	
235 min	50	178	2200	400	75	< 50
	100	335		600	77	
	200	449				
Benzo(a)pyrene	0.5 μ g	477				

* Concurrent positive control.

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sliding door: one for smoking with 20 seats and one for non-smoking with 60 seats. The high responses in the smoking compartment are not unexpected and a dependence on the number of cigarettes smoked is evident. Most samples collected in the non-smoking compartment have a relatively low response but there is one exception in the sample collected 870215. This train was congested and there was much passenger movement with frequent openings of the door: the smell of tobacco smoke in the non-smoking compartment was apparent.

Samples have been collected during 2 short air flights and 2 transatlantic flights (Table 3). The short flights gave only samples sufficient for 1 assay with 1 plate per dose but even with this

limitation a dose-dependent increase can be observed resulting in relatively high responses. There is a substantial difference between the two transatlantic flights which may be explained by the fact that the sample of 861029 was collected in a non-smoking seat only 2 rows apart from smoking seats whereas the sample of 870516 was collected in an entire non-smoking section separated from smokers by the stewardess' areas.

Experimental tobacco smoke

The experimental cigarette sidestream smoke (Table 4) was generated from a common Swedish filter brand which in this experiment gave 29.6 mg tar particles/cigarette. This sample was used for comparing the response in the regular assay and

TABLE 2

MUTAGENIC ACTIVITY AND RESPONSE IN TA98+S9 OF AIRBORNE PARTICULATE MATTER COLLECTED DURING TRAIN TRAVEL IN SMOKING AND NON-SMOKING COMPARTMENTS

Sample, date and duration	Dose (litre/plate)	Counts (rev./plate)	Response (rev./m ³)
Smoking	0	48	
860921	71	78	
250 min	143	126	600
5 cig. smoked	286	226	
Benzo(a)pyrene ^a	0.5 µg	478	
Smoking	0	62	
870208	75	372	
275 min	150	705	3500
25 cig. smoked	300	929	
Benzo(a)pyrene	0.5 µg	477	
Non-smoking	0	48	
861002	71	49	
250 min	143	60	100
	286	92	
Benzo(a)pyrene	0.5 µg	478	
Non-smoking	0	53	
870121	75	61	
270 min	150	75	100
	300	83	
Benzo(a)pyrene	0.5 µg	444	
Non-smoking ^b	0	53	
870215	50	79	
240 min	100	117	600
	167	147	
Benzo(a)pyrene	0.5 µg	427	

^a Concurrent positive control.

^b 2 additional non-smoking samples collected 870210 and 870219 gave responses of 100 rev./m³.

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the presently employed microsuspension assay and for studies of the response of the basic and non-basic fractions of the smoke. For comparison, a sample of urban airborne particulate matter was also investigated and fractionated simultaneously.

The total response of the sidestream smoke obtained in the present study in the microsuspension assay is higher than previously reported values (Ling et al., 1987). This is mainly due to the use of the modified assay technique. Two other experimental cigarette sidestream samples were analyzed in the course of this study and the assays gave 220 000 and 290 000 revertants/cigarette with TA98 in the presence of S9.

The much higher response of the microsuspension assay (Table 4), known from previous investigations (Kado et al., 1983, 1986; Ling et al., 1987), is evident for both the sidestream smoke and the urban particulate sample.

In the plate incorporation assay, the response in TA98 + S9 of the basic fraction of sidestream

smoke is about 67% of the total response. This is in agreement with earlier studies in which 67 and 70% were obtained (Löfroth et al., 1983; Löfroth and Lazandis, 1986). In the microsuspension assay, the response of the basic fraction is about 45% of the total response in TA98 + S9. This lesser relative response of the basic fraction in the microsuspension assay is further supported by the results obtained with 2 environmental samples. The first sample was collected in an apartment during a party and gave a response of 3200 rev./m³ (Ling et al., 1987) and showed after fractionation that 45% was present in the basic fraction (data not shown). The second sample, collected in the non-smoking section (corner) of a coffee shop in downtown Stockholm (data not shown), had about 36% of the total response of 2200 rev./m³ in the basic fraction. A contribution of about 400 rev./m³ from ambient outdoor particulate matter would in this case explain the fractionation result.

Urban airborne particulate matter has very lit-

TABLE 3
MUTAGENIC ACTIVITY AND RESPONSE IN TA98 + S9 OF AIRBORNE PARTICULATE MATTER COLLECTED DURING AIR TRAVEL IN NON-SMOKING SECTIONS

Sample, date, flight and duration	Dose (l air, plate)	Counts (rev., plate)	Response (rev. m ⁻³)
Gothenburg-Oslo	0	52	
860919	25	66	
SK886	50	78	500
38 min	75	91	
Oslo-Stockholm	0	52	
860919	25	70	
SK708	50	96	1000
38 min	75	143	
Benzo(a)pyrene ^a	0.5 µg	467	
New York-Oslo	0	46	
861029	24	65	
SK902	48	82	800
360 min	96	117	
	192	193	
Benzo(a)pyrene	0.5 µg	403	
New York-Stockholm	0	68	
870516	50	87	
SK904	100	96	200
390 min	200	111	
Benzo(a)pyrene	0.5 µg	634	

^a Concurrent positive control.

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TABLE 4

MUTAGENIC RESPONSE IN TA98 OF UNFRACTIONATED AND FRACTIONATED EXTRACTS OF CIGARETTE SIDESTREAM PARTICULATE TAR AND AN URBAN PARTICULATE MATTER SAMPLE IN THE REGULAR PLATE INCORPORATION ASSAY AND THE MICROSUSPENSION ASSAY AND THE ACTIVITY OF CONCURRENT POSITIVE CONTROL COMPOUNDS

Sample	Unit	Plate incorporation			Microsuspension	
		- S9	+ S9-4%	+ S9-10%	- S9	+ S9-10%
<i>Sidestream smoke</i> ^a	Rev./cig.					
Crude extract		2700	- ^c	37000	70000	240000
Basic fraction		1000	-	25000	26000	110000
Non-basic fraction		1500	-	5400	19000	75000
<i>Urban particulates</i> ^b	Rev./mg					
Crude fraction		450	520	-	3000	4200
Basic fraction		20	30	-	100	120
Non-basic fraction		460	430	-	2700	2200
<i>Positive controls</i>	Rev./plate					
5.0 µg quercetin		-	-	-	257	-
25.0 µg quercetin		318	-	-	-	-
0.5 µg benzo[a]pyrene		-	-	-	-	267
2.5 µg benzo[a]pyrene		-	385	207	-	-
<i>Spontaneous control</i>	Rev./plate	26	33	36	26	36

^a The responses have been evaluated with 6 doses in the range 0.001-0.1 cig./plate in the plate incorporation and in the range 0.00025-0.024 cig./plate in the microsuspension assay.

^b The responses have been evaluated with 6 doses in the range 25-1600 µg./plate for both assays.

^c -, not tested.

the activity in the basic fraction (Table 4) which is in agreement with earlier results (Löfroth, 1981; Löfroth et al., 1983).

Discussion

Public indoor locations

This exploratory study shows that typical public indoor locations, a shopping center plaza and a railway station waiting room, are much more polluted than the ambient outdoor air by mutagenic compounds present in airborne particulate matter. Although the sampling and analysis cannot prove the origin of the increased mutagenic activity, smoking is the only conceivable source. Concomitant with an increase of the mutagenic response there is consequently also an increase of other pollutants, such as nitrogen oxides and volatile hydrocarbons which are not detected by the mutagenicity test. The level of the indoor mutagenic response of 1000-2500 revertants/m³ (Table 1) can be compared with an average response of 45 revertants/m³ (range 9-162) for seventy-six 24-h

samples collected at street level at various locations in Gothenburg (Sweden) and analyzed with the microsuspension assay with TA98 + S9 (Löfroth et al., unpublished results). The ambient outdoor response of < 50-260 revertants/m³ measured simultaneously in the present study (Table 1) is of a reasonable magnitude considering the fact that these samples were collected during a few hours in the afternoon when the traffic is of more than the average 24-h intensity.

Train travel

The mutagenic responses of the air of smoking train compartments (Table 2) are of an expected magnitude. The type of compartment sampled has a volume of about 40 m³ and assuming an efficient air mixing, the concentration of 600 and 3500 revertants/m³ following smoking of 5 and 25 cigarettes, respectively, during 4-4.5 h may be obtained with a combined ventilation and surface removal rate (Repace and Lowrey, 1980) corresponding to about 9-12 air changes/h.

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The result from the non-smoking compartments (Table 3) shows that smoke from the smoking compartment can penetrate into the non-smoking section. Although most samples only gave a small response, an activity of 100 revertants/m³ must be judged to be above the background as the electrically powered train mostly travels through rural areas. Separation of non-smoking and smoking compartments in a train is most easily done by having an entire smoking car (or several) at the rear end of the train.

Air travel

Tobacco smoke in airplanes has recently been studied with nicotine analysis by Oldaker and Conrad (1987) who found that the average nicotine concentration in the non-smoking sections was about half of that in the smoking sections: 5.5 vs. 9.2 µg/m³. The concentration in the non-smoking sections corresponds to about 0.0013 cigarettes/m³ using the sidestream emission of nicotine (4.1 mg/cigarette) given by Rickert et al. (1984). The mutagenic response of 200–1000 revertants/m³ (Table 3) obtained in the present study corresponds to 0.001–0.005 cigarettes/m³ of which the higher value best relates to the situations investigated by Oldaker and Conrad, i.e. non-smoking seats near smoking sections.

The results of this study and of the investigation by Oldaker and Conrad (1987) indicate that if smoking is permitted on airplanes, the smoking section ought to be physically separated from the non-smoking sections, e.g. by stewardess' areas.

Chemical fractionation

Using the plate incorporation assay, previous fractionation studies have shown that mutagens in tobacco tar particulates to a large extent are basic compounds responsible for more than 65% of the response. The relative activity of the basic fraction in the microsuspension assay is smaller with slightly less than 50% of the total response being recovered in this fraction (Table 4). This is, however, still significantly more than the corresponding relative response of compounds in ambient particulate matter with a very small contribution from the basic fraction. There are no published reports indicating that some commonly occurring processes generate airborne particulate matter with

a high portion of the mutagenic activity in the basic fraction. A conceivable source of basic mutagenic compounds is cooking but it has only given a weak correlation to the total indoor mutagenic activity (van Houdt et al., 1984; Lewtas et al., 1987). The sensitive microsuspension assay might thus be used as a tobacco-specific analysis if part of an air particulate sample is subjected to fractionation, as has been explored in this study with analysis of a sample from a restaurant and a sample from an apartment (see Results). Such differential analyses are deemed possible for moderately tobacco smoke-polluted air with sample sizes 2–3 times larger than those used in the present study of public indoor locations.

Mutagenic activity and other tobacco smoke indicators

Nicotine has so far been the only tobacco-specific air pollutant. Its value as an indicator may, however, be limited as nicotine may not be a health issue and as it may be prone to rapid adsorption to surface materials causing an underestimate of the air pollution of other smoke components. Among other compounds emitted in the sidestream, several unsaturated hydrocarbons have high emission factors (Löfroth et al., 1987, and unpublished data). The emission of isoprene is about 2–3 mg/cigarette and this alkadiene may be utilized as a semi-specific tobacco smoke indicator although it is present at low background concentrations originating from natural sources (Gelmont et al., 1981; Lamb et al., 1986). An advantage with isoprene, which it shares with mutagens in the tar particulates, is that it is a potential mutagen and carcinogen following mammalian metabolism (Longo et al., 1985). Ultimately, a combination of *Salmonella* mutagenicity of particulates, isoprene and nicotine may be used for a better estimate of environmental tobacco smoke.

Acknowledgement

This study was partly made feasible by the Cooperative Agreement CR812935-01 between the U.S. Environmental Protection Agency and the Nordic School of Public Health covering a DVS program for GL.

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Highly Sensitive Methods for the Evaluation of Carcinogens and Mutagens Indoor

H. Matsushita, K. Tanabe, and S. Goto

Summary

This paper deals with low noise samplers for collecting particulates indoors, and analytical and biological methods sensitive enough to determine carcinogens and mutagens indoors. Two types of low noise samplers were devised. One can collect airborne particulates on a filter at a flow rate of up to 25 l/min. The other is a 12 stage Andersen low-pressure impactor sampler for particle size distribution studies operated at a flow rate of 20 l/min. Noise level of these samplers was less than 50 dB. Polynuclear aromatic hydrocarbons (PAH) and nitroarenes in the particulate samples collected by these samplers were determined by the following highly sensitive analytical methods:

- 1) PAH analysis which consists of ultrasonic extraction and high performance liquid chromatography (HPLC) with spectrofluorometric detector, and
- 2) nitroarene analysis which consists of ultrasonic extraction, fractionation of nitroarenes by absorption HPLC, chemical reduction of nitroarenes to the corresponding aminoarenes, and separation analysis by HPLC/fluorometry.

Concentration of PAH and nitroarenes in a smoking room were usually higher than those in a non-smoking room, and most of these chemicals were found in particulates smaller than 1 μm in particle size. We confirmed that the micro-forward mutation assay using *Salmonella typhimurium* strain TM677 was 10 times or more sensitive than the Ames method. This bioassay was useful for the measurement of mutagenic activity of particulates indoors and revealed that mutagenic activity in a smoking room was usually higher than that in a non-smoking room. Furthermore, we developed a highly sensitive method for analyzing PAH in particulates, collected by a personal sampler. This method consists of ultrasonic extraction, liquid-liquid partition, and separation analysis by column concentration/HPLC/fluorometry. This method is suitable for analysis of benzo(a)pyrene, benzo(k)fluoranthene and benzo(ghi)perylene in a particulate sample collected by only 300 l-air sampling. Some results on personal exposure to these PAH are also presented.

Introduction

Recently, carcinogens and mutagens indoors have attracted much attention for the following reasons:

- 1) We spend 80 percent or more of our time indoors [1].
- 2) Indoor air is polluted not only by pollutants in the outdoor air penetrating the indoor space but also by pollutants from indoor emission sources such as smoking, cooking, heating etc.

H. Kasuga (Ed.) Indoor Air Quality
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- 3) All these pollutants contain various kinds of carcinogens and mutagens.
- 4) Recently, indoor ventilation rates have become lower than in the past due to advances in construction techniques and growing concern with energy saving. Thus, pollutants from indoor emission sources are increasing in their weights in indoor pollution. In fact, several studies show that concentrations of carcinogens, mutagens and related pollutants in a room, where there is being heated, cooked and/or smoked, are higher than those outdoors [2-7].
- 5) The lung cancer mortality rate is increasing in major countries of the world.
- 6) There is growing consensus that the indoor air quality is very important for a healthy and comfortable life, and that this is basically controllable because indoor space is generally small and many kinds of techniques for air quality control have been developed during the past years.

However, little information has been published about carcinogens and mutagens indoors. This is mainly due to the fact that the techniques, for the survey of carcinogens and mutagens indoors have not been fully developed through sampling, bioassay and chemical analyses.

Carcinogens and mutagens indoors are divided into gaseous/vaporous ones and particulate ones. We developed several techniques for measuring carcinogens and mutagens in particulates suspended in indoor space. That is, two kinds of low noise air samplers for collecting particulates indoors, a microforward mutation assay which can measure mutagenicity of particulates collected by the low noise samplers, and highly sensitive chemical analyses for polynuclear aromatic hydrocarbons (PAH) and nitroarenes have been developed, and these techniques have been applied to evaluate indoor pollution. This paper describes the outline of these technique and gives some examples of the application of these techniques to measure carcinogens and mutagens indoors.

Development of Low Noise Samplers

Prior to the measurement of carcinogens and mutagens in particulates indoors, the particulates should be collected by a sampler which should meet the following requirements; i) low noise, ii) large sampling flow rate in the range which does not affect the pollution level indoors, and iii) small in size and portable. These requirements are necessary to survey indoor pollution without disturbing the inhabitants of the studied buildings. We developed two types of low noise samplers which satisfied these requirements. The first one is a size-dependent sampler which can collect particulates in 12 stages according to their particle sizes. The deposition rate of particulates into the lung is largely affected by the particle size. Therefore, this sampler will offer useful information about the particle size distribution of carcinogens and mutagens indoors. The other sampler is a low noise sampler which is small in size and can collect particulates without differentiation for size. This sampler is useful for survey of indoor pollution.

The Size-Dependent Sampler

Concentrations of carcinogens and mutagens in indoor air are generally very low. Therefore, the flow rate of the sampler should be as large as possible without disturbance of the indoor pollution level. In Japan, living rooms usually have an 8-mat area. The air volume of an 8-mat room is about 32 m³. Supposing that the natural ventilation rate in a

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living room is 0.5 times per hour and that the permissible extent of disturbance of the indoor by sampling is 10 percent or less of the natural ventilation rate, the maximum sampling flow rate is calculated to be about 27 l/min. Hence, we decided to develop an impactor type size-dependent sampler with a flow rate of 20 l/min, which can collect particulates separately in 12 stages according to the following 50% cut-off particle diameters; 12.1 μm or more, 8.5 μm , 5.7 μm , 3.9 μm , 2.5 μm , 1.25 μm , 0.76 μm , 0.52 μm , 0.33 μm , 0.22 μm , 0.13 μm , and less than 0.13 μm .

A powerful suction pump was needed to operate this sampler, because the pressure loss of the sampler was -377 mmHg. Various kinds of pumps and various sound insulation methods were studied in order to develop a small, low noise size-dependent sampler. As a result, we found that with the Gast DAA-103GB pump housed in a sound-proof, anti-vibration case (465 (H) \times 400 (W) \times 660 (D)), made of steel, double structure) with castors, the noise level can be reduced to 50 dB or less even when airborne particulates indoors were collected in the 12 stages at a flow rate of 20 l/min. The sampler can be operated continuously for as long as two weeks or more.

Low Noise Sampler

Pressure loss in the sampler is not large when it is used for the collection of particulates without differentiating for size. Therefore, size reduction of the sampler is feasible. We devised the following sampler by putting the Nittoh Koki VP-0935 Pump with a suction flow rate of 60 l/min under no load in a vinyl chloride case (320 (H) \times 300 (W) \times 420 (D)) with castors, and packing sound absorbing materials around the pump. This sampler can collect airborne particulates in the flow rate of 5-25 l/min. Noise level was less than 50 dB even when sampling was carried out at the flow rate of 25 l/min. This sampler can operate continuously without any trouble for two weeks or more.

We also devised pocket low noise samplers with the flow rate of 2 l/min and 0.1 l/min, respectively. The latter sampler is about 400 g in weight and can be used as a personal sampler.

Development of a Highly Sensitive Mutation Assay and Its Application to Indoor Pollution Survey

Mutagenicity of particulates in the environmental air has been extensively surveyed by the Ames methods [8-21]. This method can be used only for the measurement of the mutagenicity of particulates collected from 500 m³ or more of environmental air. Thus this method has no adequate sensitivity to measure the mutagenicity of indoor particulates which are hard to collect in large quantities. We made efforts with Dr. Lewtas, from the U.S.EPA, to develop a highly sensitive and relatively simple mutation assay which permits to measure mutagenicity of particulates indoors [2, 22]. Figure 1 shows the scheme of this micro-forward mutation assay.

The low noise sampler described before was operated at a flow rate of 20 l/min for 24 h each time, and airborne particulates were collected indoors on a quartz fiber filter (Pallfex, QAST2500). The organic compounds in the particulates were extracted by the ultrasonic extraction methods using benzene-ethanol (3:1, v/v) as an extracting solvent [23]. The extracted solution was dried under reduced pressure at 32-35°C and the residue was dissolved in 2 ml of benzene. Aliquots of the benzene solution (1,000, 500, 250 and 100 μl) were transferred in small test tubes, 2 μl of DMSO was added to each tube, and the

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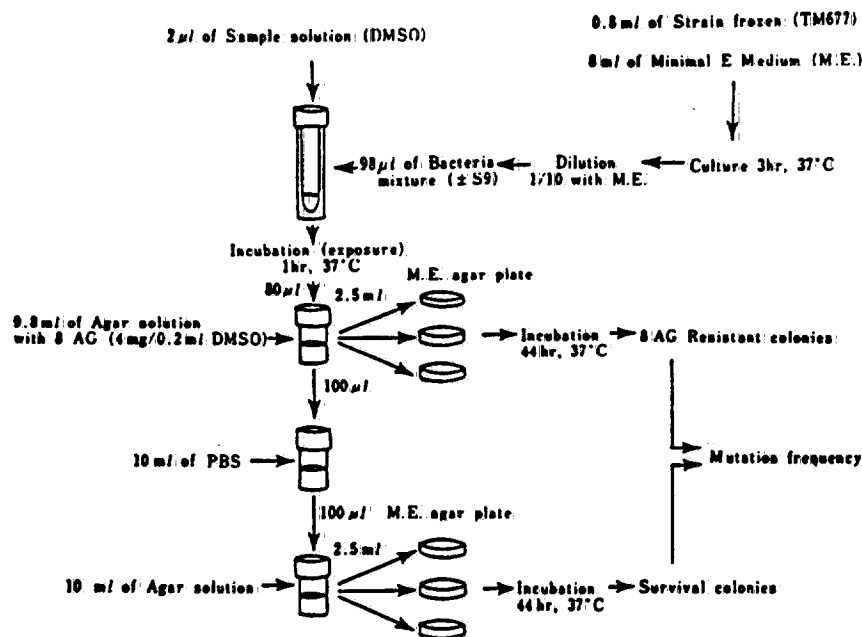


Fig. 1. Scheme of the micro-forward mutation assay.

benzene was evaporated under a gentle flow of nitrogen gas at room temperature. Then 98 μ l of a solution containing *Salmonella typhimurium* TM677, which was prepared by the 10 times dilution of the fluid cultured for 3 h at 37°C, with Minimal E medium, was added to the test tubes, and the solutions were preincubated for one hour at 37°C. After that, 80 μ l of the incubated solution was added to 10 ml of soft agar containing 4 mg of 8-azaguanine (8-AG), mixed well, and 2.5 ml portions of the agar were poured into each of three M.E. agar plates, and spread uniformly. The plates were incubated for 44 h at 37°C, and the number of 8-AG resistant colonies induced was counted.

On the other hand, survival colonies were measured by the following procedures. 100 μ l of the remaining soft agar was diluted 10,000 times in two steps, and 2.5 ml portions of the diluent were poured into each of three M.E. agar plates, and spread uniformly. The plates were incubated for 44 h at 37°C and the number of survival colonies were counted. The mutation frequency was calculated from the number of 8-AG resistant colonies and the number of survival colonies.

Figure 2 shows the dose-response curves for mutagenicity of an airborne particulate extract by the micro-forward mutation assay described above. The extract was prepared from outdoor airborne particulates collected by a high volume sampler. Figure 2 also shows the results of analysis using the Ames method with *Salmonella typhimurium* TA98 and TA100 with and without S9 mix. This figure demonstrates clearly that the micro-forward mutation assay has a far higher sensitivity than the Ames method. Furthermore, the forward mutation assay needs only one tester strain of *Salmonella typhimurium* TM677, whereas the Ames method needs several kinds of tester strains in the mutagenicity assay of chemicals, airborne particulates and so on. It was estimated

Fig. 2. Comparison of mutagenic responses for airborne particulate extracts by the micro-forward mutation assay and the Ames Method.

Micro-forward mutation assay
 O: +S9 mix ●: -S9 mix
 Ames method (Pre-incubation assay)
 □: +S9 mix ■: -S9 mix for TA100
 △: +S9 mix ▲: -S9 mix for TA98

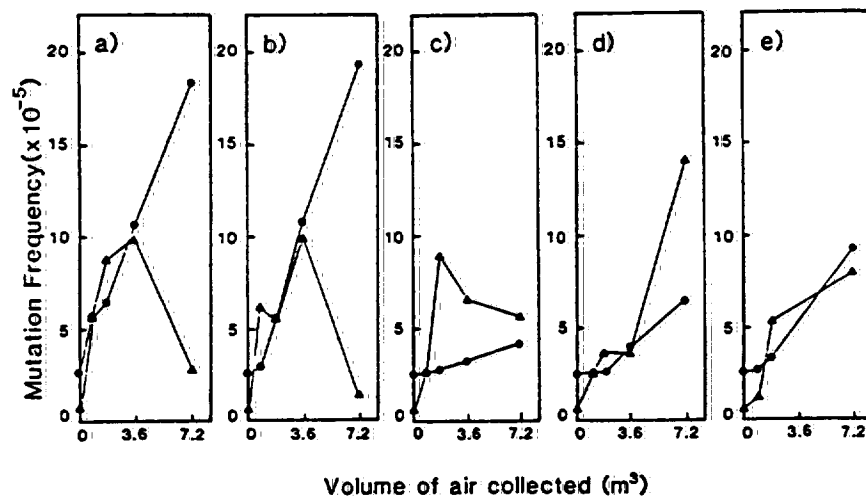
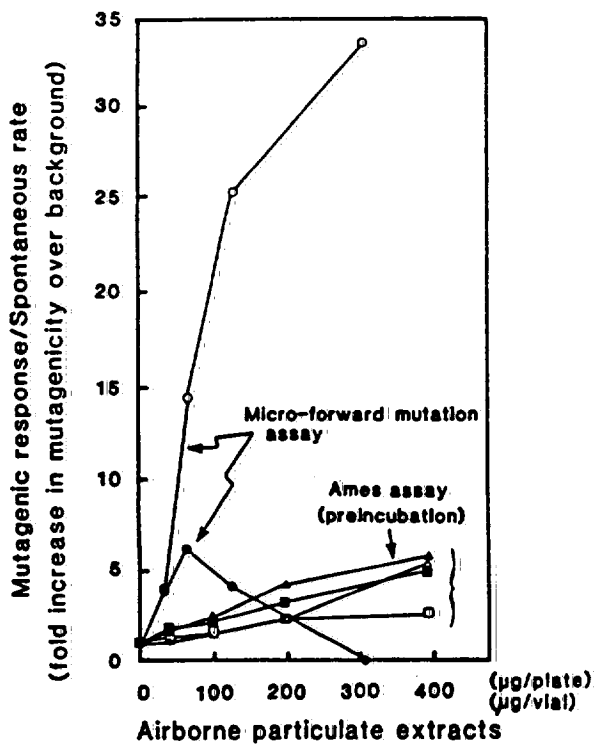


Fig. 3a-e. Dose-mutagenic frequency relationship for indoor and outdoor airborne particulate extracts. a-d: Indoor samples from the room with a smoking, b smoking and boiling water on a portable gas stove, c non-smoking and d non-smoking and handling a gas burner. e Outdoor sample. ●: +S9 mix ▲: -S9 mix

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from these facts that the effective sensitivity of the micro-forward mutation assay was 10 to 20 times higher than the Ames method. Relative standard deviation of the mutagenic activity of airborne particulate extracts in the micro-forward mutation assay was less than 12%, showing that the repeatability of this assay was equivalent to that of Ames method.

Figure 3 shows an example of a dose-mutagenic frequency relationship for indoor and outdoor airborne particulate extracts. All airborne particulates were collected by the low noise samplers at a flow rate of 20 l/min for 24 h on the same day in our Institute (air sampling volume: 28.8 m³). It can be seen from Fig. 3 that the air in a smoking room is apparently more mutagenic than the air in a non-smoking room. The mutagenicity of the air in a non-smoking room where no gas burner is used shows the same pattern as the mutagenicity of outdoor air.

Airborne particulates in a smoking room were collected by the size-dependent sampler at a flow rate of 20 l/min for 3 days, and the mutagenic activity of the extracts from the particulates collected in each stage has been measured by the micro-forward mutation assay. Table 1 shows the results. Mutation frequency per cubic meter of air was 34×10^{-5} under the test condition with S9 mix, and 51×10^{-5} under the test condition without S9 mix. Mutagenic activity of the particulates of 5.7 μ m or less accounted for 91% of the total activity under the test condition with S9 mix, and for 94% of the total activity under the condition without S9 mix. Particulates of 1.25 μ m or less in particle diameter have a fairly high-deposition rate into the deep part of the lung. Mutagenic activity of these fine particulates accounted for 76% and 83% of the total mutagenic activity in the test conditions with and without S9 mix, respectively. These results demonstrate clearly that the majority of mutagens in indoor particulates are in the small particulates that have a high lung deposition rate.

Table 1 shows also the mutation frequency per μ g of particulates. The values were generally high in the particulates less than 1 μ m in size. This was remarkable for the

Table 1. Particle size dependency of specific mutagenic activity of airborne particulate indoors

Particle size (μ m)*	Particle concentration (μ g/m ³ , air)	Mutation frequency ($\times 10^{-5}$) per m ³ , air		Mutation frequency ($\times 10^{-5}$) per particle amount (μ g)	
		+S9 mix	-S9 mix	+S9 mix	-S9 mix
> 12.1	8.0	1.7	1.1	0.21	0.14
8.5	8.3	1.4	2.1	0.17	0.25
5.7	8.0	2.5	4.3	0.31	0.54
3.9	7.2	0.6	0.8	0.08	0.11
2.5	6.4	2.1	0.6	0.33	0.09
1.25	9.1	2.0	5.3	0.22	0.58
0.76	11.0	2.8	5.5	0.25	0.50
0.52	19.0	6.9	9.0	0.36	0.47
0.33	7.8	4.4	12.5	0.56	1.60
0.22	6.7	1.5	2.5	0.22	0.37
0.13	3.1	1.1	1.1	0.35	0.35
< 0.13	4.6	7.4	6.2	1.61	1.35

*50% Cut off size.

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Table 2. Detection wavelengths and detection limits for PAH and their recoveries from airborne particulates

Compounds	Detector wavelengths (nm)		Detection limit (ng)*	Recovery from airborne particulates	
	Excitation	Fluorescence		Recovery (%)**	C.V. (%)***
Fluoranthene	360	463	0.04	103	4.9
Pyrene	340	395	0.05	102	3.9
Chrysene	272	364	0.04	101	4.0
Benzo(e)pyrene	335	379	0.2	107	3.7
Perylene	413	474	0.008	103	2.9
Benzo(k)fluoranthene	370	406	0.03	102	5.9
Benzo(a)pyrene	370	406	0.01	99.2	5.0
Benzo(ghi)perylene	385	419	0.05	103	3.9
Indeno(1,2,3-cd)pyrene	388	508	0.1	102	2.0
Coronene	305	428	0.06	100	5.0

*S/N = 2; **Average of 10 runs; ***C.V.: Coefficient of variation.

mutagenicity obtained in the test condition without S9 mix. These results suggest that small particulates that have a high lung deposition rate are more mutagenic than large particulates in the indoor environment.

Development of Highly Sensitive Chemical Analyses and Their Application to Indoor Pollution Survey

A large part of carcinogens and mutagens are contained in the neutral fraction of the extract from airborne particulates [11, 17, 18]. Major carcinogens and mutagens in the neutral fraction are polynuclear aromatic hydrocarbons (PAH) and nitroarenes [20, 24-28]. We have developed several analytical methods for these compounds.

The first one is the major PAH analysis which can analyze easily about 10 PAH indoors [29]. This method consists of the following procedures; collection of indoor particulates by a low noise sampler, ultrasonic extraction of PAH using benzene-ethanol (3:1, v/v) as an extracting solvent, liquid-liquid partition between the extract solution and 5% sodium hydroxide aqueous solution, and separation analysis of PAH by a high performance liquid chromatography (HPLC) with spectrofluorometric detection. HPLC was carried out in the following conditions: Column; ODS column (Number of theoretical plates for benzo(a)pyrene: 8700), Mobile phase; acetonitrile-water (65:35 (v/v) for the first 10 min, 65:35-90:10 (v/v) for the 2nd 10 min, and 90:10 (v/v) for the last 10 min), Column temperature; 30°C, Flow rate of mobile phase; 1.0 ml, Detector; spectrofluorometer.

Table 2 shows the spectrofluorometric conditions for detecting the 10 PAH and their detection limits in this method. Among these PAH, chrysene, benzo(k)fluoranthene, benzo(a)pyrene and indeno(1, 2, 3-cd)pyrene are carcinogenic, and fluoranthene, pyrene, benzo(e)pyrene and benzo(ghi)perylene are cocarcinogenic in experimental animals. The detection limits indicate that even a trace amount of PAH can be detected easily. Table 2

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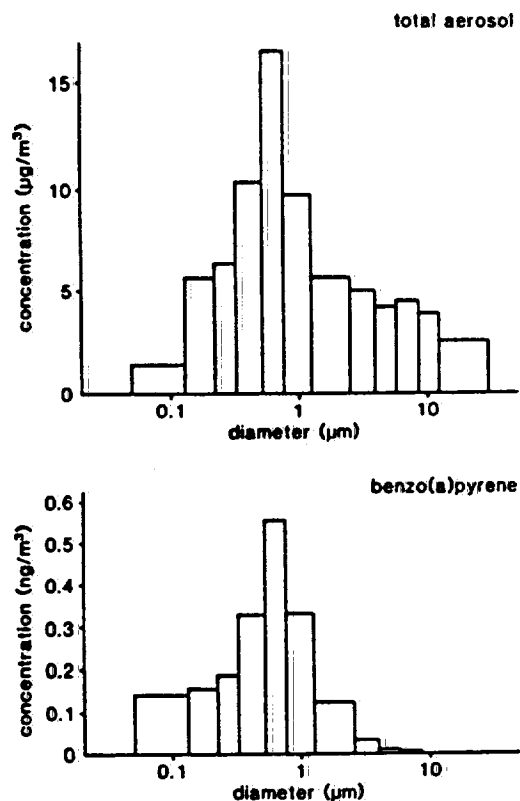


Fig. 4. Size distribution of airborne particulates (total aerosol) and benzo(a)pyrene (BaP) concentrations in indoor air

also shows recovery of PAH from airborne particulates in this method. The recovery for the 10 PAH ranged from 99.2 to 107% and the coefficient of variation from 2.0 to 5.9%. These results indicate clearly that this method is useful for the PAH analysis indoors.

This method was applied to the measurement of particle size distribution of PAH indoors. Figure 4 shows the distribution patterns for particulates and benzo(a)pyrene. Almost all of the benzo(a)pyrene is located in the particulates less than 1 µm in size, in contrast with the distribution of particulates as shown in Fig. 4. The distribution patterns for the other PAH were nearly the same as that for benzo(a)pyrene. This result shows that carcinogenic and mutagenic PAH indoors are present in the small particulates which have a high deposition rate into the deep parts of the lung.

The second method developed is for nitroarene analysis. This method consists of collection of particulates by a low noise sampler, ultrasonic extraction, separation of the neutral fraction by liquid-liquid partitions, isolation of the nitroarene fraction by a normal phase HPLC, chemical reduction of the nitroarenes to corresponding aminoarenes by sodium hydrosulfide, and separation analysis by reverse phase HPLC/fluorometry [30]. By this method, 6 carcinogenic nitroarenes can be analyzed with the following detection limits; 1 pg for 1,6-dinitropyrene, 2 pg for 1-nitropyrene, 1,3- and 1,8-dinitropyrenes, respectively, 4 pg for 2-nitrofluorene, and 40 pg for 3-nitrofluoranthene.

Table 3 shows concentration of some of the PAH and nitroarenes in smoking and non-smoking rooms. Sampling was carried out in 4 different days with low noise samplers at a

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Table 3. Concentration of PAH and nitroarenes in non-smoking and smoking rooms

Compound	Concentration in indoor air ($\mu\text{g}/\text{m}^3$)						
	1	2	3	4	Min.	Max.	Average
<i>(1) Non-smoking room</i>							
1-Nitropyrene	0.069	0.006	0.094	0.111	0.006	0.111	0.070
1-Nitrofluorene	0.011	0.005	0.007	0.004	0.004	0.011	0.007
Benzo(k)fluoranthene	0.91	0.55	0.56	1.88	0.55	1.88	0.98
Benzo(a)pyrene	1.47	1.07	1.33	4.35	1.07	4.35	2.06
Benzo(ghi)perylene	2.42	1.36	1.51	4.89	1.36	4.89	2.55
Airborne particulates*	31.3	26.6	35.6	46.9	26.6	46.9	35.1
<i>(2) Smoking room</i>							
1-Nitropyrene	0.106	0.215	0.238	0.124	0.106	0.238	0.171
2-Nitrofluorene	0.020	0.032	0.029	0.023	0.020	0.032	0.026
Benzo(k)fluoranthene	1.29	3.36	0.87	2.24	0.87	3.36	1.96
Benzo(a)pyrene	2.82	7.41	2.59	4.99	2.59	7.41	4.45
Benzo(ghi)perylene	3.34	7.90	2.22	5.17	2.22	7.90	4.66
Airborne particulates*	92.6	202	105	99.5	92.6	202	125

* unit = $\mu\text{g}/\text{m}^3$

Sampling of airborne particulates was carried out with low noise samplers at a flow rate of 20 l/min for 24 h in each 4 different days.

flow rate of 20 l/min, respectively. It can be seen from this Table that concentrations of particulates, PAH and nitroarenes in smoking room were 3.5 times, ca. 2 and ca. 3 times higher than those in non-smoking room, respectively.

Measurement of the personal exposure level to carcinogens and mutagens is important in the study of health effects from air pollution. However, determination of carcinogens and mutagens in particulates collected by a personal sampler was extremely difficult, because the amounts of particulates collected by a personal sampler is as low as several tens μg , even if the sampler is operated for 24 h. Thus, no report has been published on the personal exposure levels of carcinogens and mutagens as far as we know.

We developed a highly sensitive method for analyzing three kinds of PAH in particulates collected by a personal sampler [31]. This method consists of the following procedures; ultrasonic extraction, liquid-liquid partition between the benzene-ethanol (3:1, v/v) extract solution and 5 percent sodium hydroxide solution, drying the benzene layer fraction by gentle flushing with nitrogen gas at room temperature, dissolution of the residue into 1 ml of acetonitrile, and separation analysis by a precolumn condensation/HPLC/fluorometry. This separation technique permits the introduction of several hundreds of μl of a sample solution, and thus increases the actual analytical sensitivity about 100 times as compared with a conventional HPLC. PAH that can be analyzed by this method are benzo(a)pyrene, benzo(k)fluoranthene and benzo(ghi)perylene.

Figure 5 shows a block diagram of the device used for the separation analysis. The sample solution is introduced into the sample loop D (see Fig. 5), and then the PAH in it are transferred to the concentration column F for condensation. This condensation is done by flowing acetonitrile-water (48:52, v/v) through the column for 5 min. The

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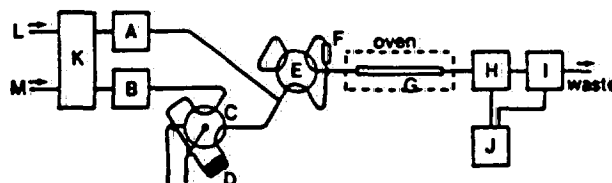


Fig. 5. Schematic diagram of the column concentration-HPLC system. A, B: HPLC pump, C: injection valve, D: Sample loop (0.5 ml), E: 6-way valve, F: concentration column (4.6 mm i.d. \times 30 mm, ODS), G: separation column (4.6 mm i.d. \times 250 mm, ODS), H: spectrofluorometer, J: UV monitor, I: chart recorder, K: degasser, L: water, M: acetonitrile:water (8:2, v/v).

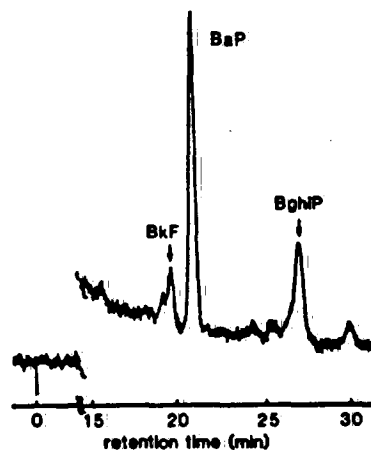


Fig. 6. HPLC chromatogram of PAH in airborne particulates collected by a personal sampler. Sampling: 200 ml/min \times 24 h

composition of the mobile phase is prepared by mixing water from pump A with acetonitrile-water (8:2, v/v) from pump B in the ratio of 4:6, respectively, immediately before the inlet of the column. After the condensation procedure is finished, pump A is stopped, and the valve E is turned in order to transfer the PAH from the column F to the column G for separation analysis.

Figure 6 illustrates a HPLC chromatogram of PAH of the extract from particulates collected by a personal sampler. Particulate samples were collected at a flow rate of 0.2 l/min for 24 hours. In this case, samples corresponding to 50 l of air were introduced into the HPLC. It can be seen from this Figure that three PAH in the personal particulate samples can be easily analyzed quantitatively by this method. The repeatability of the retention time and peak height in this method proved to be fairly good. For example, the coefficient of variation for peak height was 1.8% for benzo(a)pyrene, 2.5% for benzo(k)fluoranthene and 2.7% for benzo(ghi)perylene.

We are now surveying the personal exposure level to these PAH by the method described above. Figure 7 presents the daily variation of the exposure level to benzo(a)pyrene for four volunteers. Volunteer A and B were smokers, and C and D were non-smokers. Exposure levels to benzo(a)pyrene were generally higher in smokers than non-smokers. Furthermore, the levels changed day by day and generally decreased on holiday. The same variation pattern was seen for benzo(k)fluoranthene and benzo(ghi)perylene.

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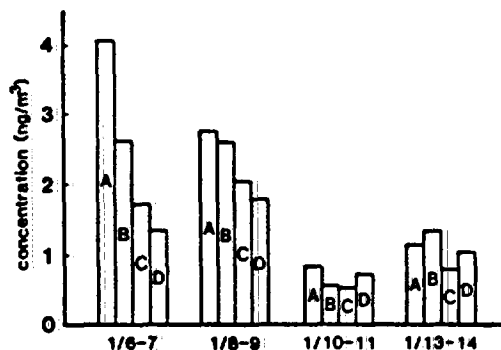


Fig. 7. Personal exposure level to benzo(a)pyrene. A and B: smoker, C and D: non-smoker. Sampling was done January 6-14, 1987. Each sampling time was 24 h (January 10-11 was a holiday)

The methods described here are effective for the measurement of mutagenicity, PAH and nitroarenes in airborne particulates indoors as well as the measurement of personal exposure levels to some PAH. Application of these methods to our living environment will produce many kinds of useful information for the evaluation of air quality indoors and also for the study on the health effects of environmental carcinogens and mutagens.

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THE EFFECT OF SMOKE AGE AND DILUTION ON THE CYTOTOXICITY OF SIDESTREAM (PASSIVE) SMOKE

(L-929 cells; cigarette design; cell death)

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(Received 3 September, 1986)

(Accepted 15 September, 1986)

SUMMARY

Decreases in the mortality of monolayer cultures of L-929 cells exposed to sidestream (passive) smoke with increases in smoke age and dilution have been reported. In the current study, the concentration of sidestream smoke (SS) to which cells were exposed was directly correlated with % mortality ($r = 0.987$) having 0% mortality at a concentration of 1.19% and a calculated mortality of 9562% at a concentration of 100%. The \ln of % mortality was correlated with increases in smoke age ($r = -0.9999$) and the regression equation was used to calculate 0 mortality at an age of 30 s and 193% mortality at the time of smoke generation. In addition, when sidestream smoke generated from a low-yield, filtered, modern design experimental cigarette was compared with that generated from a high-tar non-filtered reference cigarette, a lower number of puffs of smoke from the low-yield cigarette than from the high tar cigarette was necessary to yield 50% mortality of cells.

INTRODUCTION

A peristaltic pump smoke exposure system [1] has been used to determine the cytotoxic effects of SS smoke (passive smoke) on monolayers of murine L-929 cells [2]. The results of these studies indicated that a dose response for cytotoxicity of SS smoke existed, and that cytotoxicity of smoke decreased with increased dilution or aging of the smoke.

In a study of the effects of SS flow rates on smoke concentrations and yield of

* Presented at the International Experimental Toxicology Symposium on Passive Smoking, October 23-25, 1986, Essen (F.R.G.).

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high tar, non-filtered (2R1) and reduced-yield filtered, and tip-diluted (1R4F) reference cigarettes. Griffith, Thomas and Hughes (Abstracts of the Tobacco Chemists' Research Conference, Knoxville, TN, U.S.A., paper 51, 1986) developed and reported data which permitted an evaluation of the initial results on effects of SS smoke dilution on cytotoxicity. In addition, the results suggest the possibility that modern cigarette design may reduce the cytotoxicity of mainstream smoke, but increase cytotoxicity of SS smoke.

MATERIALS AND METHODS

The smoke exposure system

The peristaltic pump mainstream-sidestream smoke exposure system and its adaptation to study of cytotoxicity of smoke to monolayer tissue cultures has been described in detail elsewhere [1, 2]. Briefly, SS and mainstream smoke were collected as previously described [1]. Age of smoke was varied by placing 2.5-cm diameter chambers of different lengths between the smoke source and the exposed tissue culture flasks [2].

The University of Kentucky Reference cigarette, 2R1, was used in the age and dilution studies. This cigarette is a high-tar cigarette with no filter, designed to be equivalent to cigarettes in use in the 1950s [3]. The 1R4F cigarette is a reduced-yield cigarette with an acetate filter and 30% tip dilution, more porous and faster burning paper, and, therefore, approximates modern cigarette design [3]. Cytotoxic effects of mainstream and sidestream smoke from the 1R4F cigarette were compared with the effects of smoke from the 2R1 cigarette under comparable conditions.

Culture procedures

Mouse L-929 cells, a fibroblast-like cell line, were grown to a confluent state in Falcon 25 cm² tissue culture flasks (Falcon Plastics, Oxnard, CA). Culture and exposure procedures are described in detail elsewhere [2]. Control flasks of cells were subjected to all experimental conditions except exposure, and were included in all experiments. Viability of cell cultures was determined 24-48 h after exposure by means of trypan blue dye exclusion. % mortality was calculated as follows:

$$\% \text{ mortality} = \frac{(\text{control viability} - \text{sample viability})}{\text{control viability}} 100$$

Control viabilities ranged from 80-98%.

RESULTS

The effects of increases in concentrations of SS smoke reaching the exposure chamber on mortality of L-929 cells was determined (Table I). As concentration of SS smoke increased, % mortality increased (Table I). As calculated from the linear

TABLE I

THE EFFECT OF INCREASING CONCENTRATIONS OF SS SMOKE GENERATED FROM THE 2R1 CIGARETTE ON CYTOTOXICITY TO L-929 CELLS

Total SS Smoke to exposure (ml)	Concentration total SS smoke	% Mortality (% M)	Corrected for 1.68 ml/s increase in flow due to lighted cigarette	
			Concentration % total SS smoke	% Mortality (% M)
4.0	24	4	1.29	4
5.0	30	48	1.61	48
6.7	40	89	2.14	89
Linear regression equation				
		%M = (Conc.) (5.19) - 115.59	%M = (Conc.) (96.77) - 115.57	
		r = 0.99	r = 0.99	
Calculated from linear regression				
		22.3	0	0
		100	403	9562

regression curve, if the cells had been exposed to 100% of the SS smoke generated, a hypothetical 403% mortality would have been observed. These initial concentrations of SS smoke reaching the exposure chamber were calculated on the basis of total SS smoke volume. When the increase in flow rate of 1.68 ml/s for a lighted cigarette compared to an unlighted cigarette (Griffith, Thomas and Hughes, Abstracts of the Tobacco Chemists' Research Conference, Knoxville, TN, USA, paper 51, 1986) was used to calculate SS smoke concentration, exposure of the cells to 100% of the SS smoke generated would have resulted in a hypothetical 9562% mortality of the cells (Table I).

Aging of SS smoke resulted in a rapid decline in the mortality generated by the smoke (Table II). As calculated from the linear regression curve, an increase in age of SS smoke of 30 s after generation would have resulted in a total loss of cytotoxic effects (Table II).

When the cytotoxicity of smoke generated from 2R1 and 1R4F for L-929 cells was compared, interesting results were observed (Table III). When mainstream smoke was generated from the cigarettes, substantially more puffs of mainstream smoke from the 1R4F cigarette than from the 2R1 cigarette were required to induce 50% mortality of cells (Table III). However, for SS smoke, the situation was reversed. Substantially more puffs of SS smoke from the 2R1 cigarette were required to induce 50% mortality of cells than from the 1R4F cigarette (Table III).

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TABLE II

THE EFFECT OF AGING OF SS SMOKE GENERATED FROM THE 2R1 CIGARETTE ON CYTOTOXICITY TO L-929 CELLS

Smoke age (s)	% Mortality (% M)
3.8	77
6.2	28
10.2	5

Linear regression equation

$$\ln \%M = (\text{age}) (-0.4276) + 5.97$$

$$r = -0.9999$$

Calculated from linear regression

0	391
30	0

TABLE III

A COMPARISON OF THE CYTOTOXIC EFFECTS ON L-929 CELLS OF MAINSTREAM AND SS SMOKE GENERATED FROM 2R1 AND 1R4F CIGARETTES

Exposure	Value for smoke from 2R1 cigarette	Value for smoke from 1R4F cigarette
Mainstream smoke exposure (Puffs) for 50% mortality ($\bar{x} \pm \text{SEM}$)	5.8 \pm 0.6	11.4 \pm 2.4
Sidestream smoke exposure for 50% mortality ^a		
Puffs (Individual Paired Experiments)	9.1 11.5 9.0 13.1 10.9 11.0 9.3 9.8	6.2 6.7 6.6 7.0 6.6 6.6 6.4 8.6
Puffs ($\bar{x} \pm \text{SEM}$)	10.5 \pm 0.49	6.8 \pm 0.25 ^b

^a 30% Concentration of total SS smoke reached exposure chamber^b $P < 0.05$ by paired *t*-test.

DISCUSSION

The results of the present study indicate that SS smoke from the 2R1 cigarette was extremely cytotoxic to L-929 cells. When the flow rate was corrected to take into account the increase caused by the cigarette being lighted, the cytotoxic potential of the SS smoke was more clearly delineated. If as little as 1.6-3% of the SS smoke

generated from the 2R1 cigarette reached the exposure chamber containing the cells, very high levels of cytotoxicity were induced. A calculation of the toxic effects of SS smoke from the 2R1 cigarette indicated that if 100% of the SS smoke reached the cells in the exposure chamber, a hypothetical level of 9562% mortality would have been observed! This calculation of the increase in SS smoke flow when the cigarette is lighted is likely to be an underestimate of the volume of SS smoke. This would indicate that SS smoke, freshly generated from 2R1 cigarettes, was indeed very toxic.

The duration of this high level of cytotoxicity of SS smoke appears to be limited. The cytotoxic effect of SS smoke generated from the 2R1 cigarette is very high at the time the smoke is generated. However, the cytotoxic effects of the smoke progressively deteriorate with age. Calculations from the data generated indicate that by 30 s after generation of SS smoke, all cytotoxic effects should disappear. This would suggest that SS smoke is very cytotoxic, but this cytotoxic effect is lost very rapidly with aging of smoke.

The 1R4F cigarette is a filtered cigarette with 30% tip dilution, and compared to the 2R1 cigarette, has a more porous faster-burning paper and a reduced yield of tar [3]. For this reason, it was of interest to compare the cytotoxic effects of smoke generated from the 1R4F cigarette with the cytotoxic effects of smoke generated from the 2R1 cigarette. More puffs of mainstream smoke generated from the 1R4F cigarette were required to elicit 50% cytotoxicity of cells compared to mainstream smoke generated from the 2R1 cigarette. This decreased level of cytotoxicity of mainstream smoke from the 1R4F cigarette is what would be expected due to the dilution of mainstream smoke as a result of the tip dilution design of this cigarette [2].

A smaller number of puffs of SS smoke generated from the 1R4F cigarette, on the other hand, was required to generate 50% cytotoxicity than was required from SS smoke generated from the 2R1 cigarette. This demonstrates a higher level of cytotoxicity of SS smoke from the 1R4F cigarette compared with SS smoke from the 2R1 cigarette. The cytotoxic effects of smoke have been suggested to be due to, in some part, the gas phase of the smoke [2, 4-6].

Using this model system, the cytotoxic potential of SS smoke has been demonstrated. Future studies can be utilized to determine how alterations in cigarette design could alter that cytotoxic potential.

ACKNOWLEDGEMENT

This study was supported by grants from the Kentucky Tobacco and Health Research Institute. The smoke exposure equipment was provided by the University of Kentucky Tobacco and Health Research Institute. The authors thank Dr. Robert B. Griffith of the University of Kentucky College of Pharmacy for his invaluable aid in the performance of these studies and the preparation of this manuscript.

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Toxicology Letters, 35 (1987) 95-99
Elsevier

ENI 01699

DEPOSITION OF SIDESTREAM CIGARETTE SMOKE IN THE HUMAN RESPIRATORY TRACT II. DEPOSITION OF ULTRAFINE SMOKE PARTICLES*

(Aerosol; passive smoking)

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(Received 8 August, 1986)

(Accepted 1 September, 1986)

SUMMARY

Sidestream cigarette smoke was generated into an inhalation chamber from which five normal male volunteers inhaled the smoke. Size distribution of the smoke aerosol was: count median diameter, 0.11 μm , mass median diameter 0.43 μm . Deposition fraction measured as concentration difference for each size fraction between inhaled and exhaled aerosol for each size interval was: 0.075 μm , 0.24 ± 0.04 ; 0.13 μm , 0.15 ± 0.04 ; 0.24 μm , 0.10 ± 0.04 ; and 0.42 μm , 0.07 ± 0.02 . The declining deposition fraction as size approaches 0.5 μm is consistent with previous theoretical and experimental data.

INTRODUCTION

The toxicity of sidestream cigarette smoke depends in part upon the particulate dose to the respiratory tract. For mainstream cigarette smoke inhalation, deposition fraction is reported by most authors to be 0.7-0.95 [1, 2], although one recent report suggests a lower mean deposition of 0.47 [3]. These deposition fractions are higher than expected for aerosol particles in the 0.3-0.5 μm size range, which is the size

* Invited paper, presented at the International Experimental Toxicology Symposium on Passive Smoking, October 23-25, 1986, Essen (F.R.G.).

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Abbreviations: RIES, bis(2-ethylhexyl)sebacate.

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the ban. These showed that the concentrations of nicotine, carbon monoxide and particulates were virtually identical to those recorded in non-smoking compartments before the ban. The levels found on platforms, where at that time smoking was still allowed, did not noticeably change.

DISCUSSION

It was found to be possible to measure constituents of tobacco smoke in the ambient air of a public environment. The portability and inconspicuous design of the monitoring package allowed realistic measurements to be acquired. Levels observed in this study were all far lower than the recommended OSHA limits for safe exposure.

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INT. 01708

URINARY MUTAGENICITY AFTER CONTROLLED EXPOSURE TO ENVIRONMENTAL TOBACCO SMOKE (ETS)*

(Passive smoking; urinary mutagenicity; smoking; diet)

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(Received 3 September 1986)

(Revision received 23 September 1986)

(Accepted 25 September 1986)

SUMMARY

20 non-smokers on a defined diet low in polycyclic aromatic hydrocarbons (PAH) were exposed to environmental tobacco smoke (ETS) in an unventilated room for 8 h. The urinary mutagenicity in the 24-h urine samples as tested with the *Salmonella* (TA98) microsome assay did not significantly increase after exposure to either 10 ppm CO or 20-25 ppm CO. We conclude that exposure of non-smokers to ETS does not lead to an increase in their urinary mutagenicity, provided the exposure conditions are within a realistic range.

INTRODUCTION

A crude biomonitoring of acute exposure to mutagenic and potentially carcinogenic substances can be performed by measuring the mutagenicity of urine extracts. So far three studies (two on men [1,2] and one on rats [3]) have been published reporting an increase in urinary mutagenicity due to ETS exposure. Surprisingly, a low exposure of men to ETS [2] was found to cause a much higher relative increase

* Presented at the International Experimental Toxicology Symposium on Passive Smoking, October 23-25, 1986, Essen (F.R.G.).

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Abbreviations: DMSO, dimethyl sulfoxide; ETS, environmental tobacco smoke; PAH, polycyclic aromatic hydrocarbons.

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in mutagenicity than an unrealistically high exposure level [1]. It was the aim of our study to measure the mutagenicity in the urine of non-smokers before and after exposure to ETS, with the experimental conditions being strictly controlled. Furthermore, the *Salmonella typhimurium* TA98 microsome assay (Ames test) was carried out in coded samples.

MATERIALS AND METHODS

Subjects

24 healthy male subjects (10 smokers and 14 non-smokers) aged 18 to 44 years (mean age 22 years) volunteered to take part in either one or both of the following experiments.

Experiment 1

10 non-smokers were put on a controlled diet low in PAH. During the first day after admission (control day), the subjects stayed in an experimental room (45 m³, 2-3 ppm CO, 10-15 ppb NO, 20 ppb NO₂, 20 µg/m³ nicotine, 15-20 µg/m³ formaldehyde) for 8 h. Smoking was not allowed. On the second day (exposure day) the subjects were exposed to ETS (10 ppm CO, 160 ppb NO, 10-30 ppb NO₂, 40-100 µg/m³ nicotine, 40 µg/m³ formaldehyde) in the same room again for 8 h. ETS was produced by 2 smokers smoking 42 cigarettes during exposure time. Before and after each session blood samples were drawn for COHb and cotinine determination. The 24-h urine was collected for cotinine analysis and mutagenicity testing.

Experiment 2

This experiment differed from experiment 1 by the level of exposure only. Two groups of 5 non-smokers and 5 smokers each were exposed to ETS generated by the 5 smokers smoking 100 cigarettes. The levels of ETS components increased as follows (control vs. exposure day): CO: 2-3 vs. 20-27 ppm; NO: 10-15 vs. 280-350 ppb; NO₂: 15-20 vs. 130-170 ppb; nicotine: 20 vs. 120-150 µg/m³; formaldehyde: 15-20 vs. 30-50 µg/m³.

Methods

CO and NO/NO₂ were continuously recorded on a Carbon Monoxide Analyzer (Model 8310, Monitor Labs Inc., U.S.A.), and on a Nitrogen-Oxide Analyzer (Model 8840, Monitor Labs Inc., U.S.A.), respectively. Nicotine and formaldehyde were determined by capillary gas chromatography [4,5]. COHb was quantified using a CO-Oximeter (Model 182, Instrumentation Laboratories Ltd.). Cotinine in serum and urine was measured by a radioimmunoassay (RIA) [6]. Creatinine in urine was determined by the method of Jaffe [7]. For mutagenicity testing urine samples were extracted and determined according to modified methods of Yamasaki and Ames

Table I

DOSEMETRY FOR ETS EXPOSURE AND URINARY MUTAGENICITY IN EXPERIMENTS 1 AND 2: MEAN ± SD

	Experiment 1				Experiment 2				Diet-study	
	Non-smokers (n = 10)		Smokers (n = 10)		Non-smokers (n = 10)		Smokers (n = 10)		Non-smokers (n = 8)	
	Control day	Exposure day	Control day	Exposure day	Control day	Exposure day	Control day	Exposure day	Poor in PAH	Rich in PAH
COHb (%)										
before	0.34 ± 0.15	0.18 ± 0.02	***	0.63 ± 0.19	0.65 ± 0.32	***	2.44 ± 1.10	0.81 ± 0.44	***	
after	0.32 ± 0.13	0.87 ± 0.04		0.62 ± 0.13	2.69 ± 0.13		1.24 ± 0.40	7.87 ± 2.20		
Serum cotinine (ng/ml)										
before	0 ± 0	0 ± 0	***	1.2 ± 1.7	0.4 ± 1.0	***	377.8 ± 129.7	145.7 ± 51.0	***	
after	0 ± 0	1.1 ± 1.3		0.9 ± 1.6	4.9 ± 0.9		242.3 ± 84.3	244.0 ± 84.4		
Cotinine in urine (µg/24 h)	18 ± 8	23 ± 8	**	21 ± 13	67 ± 26	***	448.5 ± 179.5	358.4 ± 127.8		
Rev./plate ^a	6 - 12	3 - 16		0 - 10	0 - 19		0 - 23	15 - 376	0 - 11	14 - 25
Rev./24 h	875 ± 371	1069 ± 565	NS	236 ± 1358	548 ± 757	NS	927 ± 510	13319 ± 17224	686 ± 374	1921 ± 694
Rev./mmol creatinine	42.3 ± 18.2	56.0 ± 35.5	NS	13.1 ± 20.0	25.8 ± 38.3	NS	50.5 ± 29.4	770.3 ± 968.8	38.4 ± 18.2	133.8 ± 47.3

Statistical tests: The comparisons indicated were performed using the one-sample *t*-test for the differences (exposed - non-exposed). The results on urinary mutagenicity were tested with the Wilcoxon-Rank sum test. Levels of significance are as follows: NS, not significant; **P* < 0.05; ***P* < 0.01; ****P* < 0.001.

^aRange of highest mean rates (spontaneous rate) of triplicate measurements observed after applying 10, 25, 50 and 75 µl urine concentrate (corresponding to 2, 5, 10 and 15 ml urine).

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[8] and Mohtashamipur et al. [9]. The urine concentrates dissolved in 2 ml DMSO were investigated for mutagenicity by the *Salmonella* (TA98) microsome (S9-mix derived from Aroclor-treated rats) assay.

RESULTS

The results are summarized in Table I. COHb and cotinine in serum and urine increased in non-smokers after ETS exposure, whereas no significant rise was found in urinary mutagenicity. Using the Wilcoxon-Rank sum test, the observed increase in mutagenicity in Exp. 2 does not reach statistical significance ($P = 0.06$). It makes no difference whether the evaluation is based on the number of revertants per 24 h or on the number of revertants per mmol creatinine. The mutagenicity in urine of smokers is significantly elevated after smoking, but no correlation was found between the mutagenicity and smoking parameters such as cigarettes per day, COHb and cotinine in serum or urine. For comparison the mutagenic activities in urine after diets poor or rich in PAH are also included [10]. The urinary mutagenicity was found to increase significantly after food rich in leafy vegetables and charcoal-broiled meat.

DISCUSSION

The exposure level reached in real-life situations may correspond to that in Exp. 1 [11], although the daily exposure time is usually less than 8 h [12]. The exposure level in Exp. 2 is far from being realistic.

Bos et al. [1] exposed 8 non-smokers to the ETS of 157 cigarettes in a poorly ventilated room for 6 h. The exposure conditions were similar to those of our Exp. 2 with respect to cigarettes smoked per time unit and room volume (0.24 cig./m³/h in the Bos et al. study vs. 0.27 cig./m³/h in our study). On the day of exposure the urinary mutagenicity was found to be higher than on the days before or after exposure. The increase in mutation rates amounted to about 4% of that in smokers. This corresponds well to the rise by about 2% as found in our second experiment.

The experimental design of the study by Sorsa et al. [2] differed from ours by exposure time and exposure level, the latter being much lower than in our first experiment (4 ppm CO vs. 10 ppm CO). In spite of this, the mutagenic activity in their passive smokers increased to about 70% of that found in smokers. The extent of this relative increase is surprisingly high and certainly in contrast to the results obtained by Bos et al. [1] and by us.

As shown in Fig. 1, for both the ETS-exposed and the PAH-diet consuming subjects the number of revertants induced by the urine extracts was less than twice the spontaneous mutation rate, indicating that the criterion of a positive test result as established by Ames [8] is not met. When testing the urine of our cigarette smokers, however, the results were clearly positive demonstrating a high sensitivity of our ex-

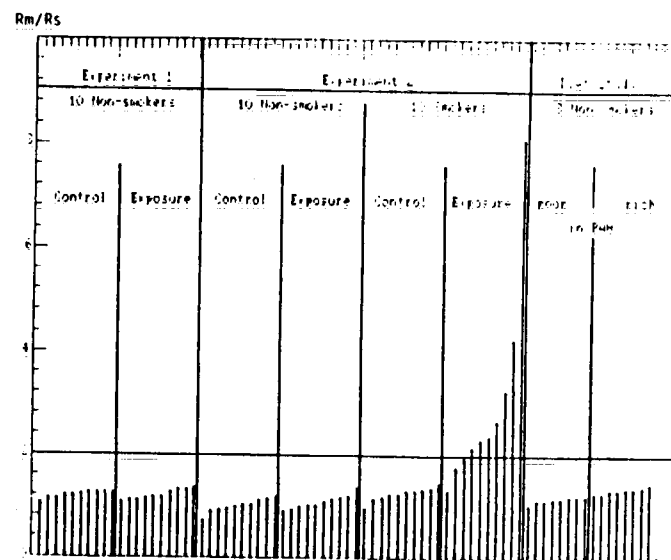


Fig. 1. Maximum number of revertants per plate derived by the spontaneous mutation rate (Rm/Rs). Individual values within each subgroup are sorted in ascending order.

traction and test procedures [8,9,13]. Furthermore, the large variability in our mutagenicity data, which was found despite strictly controlled conditions shows that yet unknown methodological and endogenous factors clearly outweigh the marginal increases in urinary mutagenicity after ETS exposure. Moreover, the relevance of an increased urinary mutagenicity with respect to cancer risk assessment is unclear [13]. These considerations support the view that measuring the mutagenic activity in passive smokers does not lead to results that predict an increased risk to human health.

ACKNOWLEDGEMENTS

The authors wish to thank Mr. H. Begutter and Dr. H. Klus for the ambient air measurements.

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INT 01709

URINARY EXCRETION OF MUTAGENS IN PASSIVE SMOKERS*

(Mutagenicity: urine; cotinine; passive smoking)

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SUMMARY

Six healthy young volunteers with no history of active smoking were asked to keep on their Western diets avoiding the consumption of alcoholic beverages, excess coffee, any sort of medication, and the known pro- and/or anti-mutagen-containing foods and drinks, 24 h before and during the experiments. They were exposed passively to cigarette smoke produced by 4 habitual smokers in an unventilated 48.6 m³ room for 8 h. The carbon monoxide concentration was 18.85 ± 7.3 ppm during the 8 h exposure.

Frameshift mutagens were isolated from 10-h urine samples using chloroform and were tested for mutagenicity in the *Salmonella*/mammalian microsome assay employing *Salmonella typhimurium* TA98. Although clearly enhanced, no significant mutagenic activity could be found with 25 ml equivalent urine/plate after passive exposure to cigarette smoke. The weak mutagenicities found were highly significant when 50 ml equivalent urine/plate was tested. No direct correlation was observed between urine mutagenicity and the urinary cotinine concentration. The results obtained are discussed with reference to inconsistent reports in the literature concerning the mutagenicity of urine after passive smoking.

INTRODUCTION

Inconsistent evidence exists in the literature concerning the recovery of mutagens excreted in urine after passive smoking. Bos et al. [1] reported, for the first time, that 12-h urine of nonsmokers staying in a smoky room for 6 h was mutagenic. In contrast, the 1985 report of Sorsa et al. [2] indicated no significant (although suggestive) difference between urine mutagenicity before and after passive smoking (their subjects were habitual smokers after a 48-72 h smoking cessation). The very recent paper of Scherer et al. [3] indicates non-mutagenicity of the urine of

* Presented at the International Experimental Toxicology Symposium on Passive Smoking, October 23-25, 1986, Essen (F.R.G.).

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Abstracts of the Twenty-Second Annual Scientific Meeting of the Environmental Mutagen Society

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ASSESSMENT OF THE BIOLOGICAL ACTIVITY OF MAINSTREAM OR ENVIRONMENTAL TOBACCO SMOKE (ETS) USING A CELLULAR SMOKE EXPOSURE TECHNIQUE. D. W. Bernicker, P. H. Ayres*, P. R. Nelson*, C. R. E. Goggins*, D. Franco*, Q. Fulp*, G. Lee and D. J. Dockline. R. J. Reynolds Tobacco Company, Winston-Salem, NC 27102.

The development of appropriate methods for exposing cell cultures to whole smoke are necessary for evaluating the biological activity of environmental tobacco smoke (ETS) in mammalian and bacterial cells. The cellular smoke exposure technique (CSET) uses a rocking apparatus that allows cells to equilibrate between exposure to a known smoke concentration and culture medium maintained at 37°C. The neutral red assay in WB rat

liver cells and an Ames assay were used to assess cytotoxicity and mutagenicity, respectively. Mainstream smoke generated from 1R4F reference cigarettes was used to characterize the CSET. WB cells were exposed for one or two hours to varying mainstream smoke concentrations (40 to 640 mg total particulate matter (TPM)/m³). The TA98 *Salmonella* bacteria were exposed for two hours to a mainstream smoke concentration of 350 mg TPM/m³. The cytotoxicity of 1R4F mainstream smoke to rat liver cells was concentration and time dependent with no observed effect levels for one and two hour exposures of approximately 180 and 40 mg TPM/m³, respectively. The TA98 *Salmonella* strain exhibited approximately a two-fold increase in the number of revertants/plate after exposure to 1R4F mainstream smoke and an 89 metabolic activation system. WB rat liver epithelial cells and the TA98 *Salmonella* bacterial strain were exposed to a relevant concentration of ETS (approximately 1.8 mg TPM/m³) for three hours. Using the neutral red cytotoxicity and Ames mutagenicity assays there were no differences observed in the ETS-exposed cells and their respective room air controls, indicating that ETS was biologically inactive as tested. The use of CSET permits determination of concentration X time relationships and threshold concentrations for the biological effects of mainstream, sidestream and environmental cigarette smoke without resorting to artificial smoke collection methods.



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BIOMONITORING OF EXPOSURE TO POTENTIALLY GENOTOXIC SUBSTANCES FROM ENVIRONMENTAL TOBACCO SMOKE

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El 87-481 (Received 5 November 1987; Accepted 5 May 1987)

In two experimental studies, 10 volunteer nonsmokers were put on a controlled diet and exposed to two different ETS concentrations for 8 hours. During exposure in Experiment 1, the average indoor air levels of CO, NO, NO₂, formaldehyde, and nicotine reached 10 μ L/L, 160 nL/L, 20 nL/L, 40 μ g/m³ and 60 μ g/m³, respectively. During Experiment 2, the respective concentrations were 20 to 25 μ L/L, 310 nL/L, 150 nL/L, 50 μ g/m³ and 120 μ g/m³. On the average, carboxyhemoglobin increased by 0.7% after exposure in Experiment 1 and by 2.0% in Experiment 2. The serum cotinine concentration increased by 1 ng/mL and 5 ng/mL in Experiment 1 and 2, respectively. On the average, the subjects excreted 24 μ g (Experiment 1) and 70 μ g cotinine (Experiment 2) in the 24-h urine after ETS exposure. No statistically significant increase was found in the urinary mutagenicity after either of the two exposure regimens, whereas thiocyanate excretion was significantly elevated. The data suggest that nonsmokers in real-life situations take up very low doses of ETS constituents, and detoxification of the genotoxic substances inhaled is effective.

INTRODUCTION

Environmental tobacco smoke (ETS) is a complex mixture of several thousand chemical compounds. These compounds are found in mainstream smoke as well, although its quantitative composition is quite different (Klus and Kuhn 1982). Dosimetry of ETS exposure should be based on biochemical markers that should be specific and representative. The commonly used markers, such as nicotine, cotinine, carboxyhemoglobin (COHb), and thiocyanate, do not meet all these requirements (for extensive review,

see U.S. Department of Health and Human Services 1986). Carboxyhemoglobin and thiocyanate (a metabolite of tobacco-smoke-derived hydrogen cyanide) were found to be inadequate biomarkers in field studies mainly because of interference with sources other than ETS. Because of its stability and specificity for tobacco smoke, cotinine in body fluids appears to be the short-term marker of choice in population studies. However, since carbon monoxide, hydrogen cyanide, and nicotine are constituents of the gas phase of ETS (Eudy et al. 1985), an adequate marker for quantitating particulate-phase exposure is lacking. Additionally, these substances are not related to the genotoxic properties of ETS, an attribute that is of highest concern with respect to

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long-term health effects of ETS exposure (IARC 1986). Solid risk assessments and comparisons to other risk factors such as smoking, diet, and ambient air pollution require dosimetric data on the uptake of toxic and genotoxic substances during ETS exposure.

The objective of the investigation was to measure urinary excretion of mutagenicity and thioethers in addition to COHb and cotinine in serum and urine after high but controlled exposures of nonsmokers to ETS. Urinary mutagenicity, which can be regarded as a measure of exposure to genotoxic substances, has been found to increase in three experimental settings (Bos et al. 1983; Sorsa et al. 1985; Mohtashamipur et al. 1987) but not in another experiment (Scherer et al. 1987) nor in a real-life situation (Husgafvel-Pursiainen et al. 1987). Thioether excretion, which can be regarded as an indicator of exposure to electrophilic substances, has been reported to increase after smoking (Van Doorn et al. 1979) but to be unchanged after ETS exposure (Sorsa et al. 1985). Interest was focused on the relationship between excretion of thioethers and mutagenicity in the urine after controlled ETS exposure, since both urinary thioethers and mutagenicity might at least partly be caused by exposure to the same substances.

SUBJECTS AND METHODS

Subjects

Twenty-four healthy male subjects (14 nonsmokers and 10 smokers) aged 18 to 44 years (mean age 22.0 years) volunteered to take part in either one or both experiments. After admission to the laboratory on a Friday evening, they completed a questionnaire on socioeconomic and life-style factors as well as on their ETS exposure during the past 48 h.

Protocol

Experiment 1. After admission to the laboratory at 8:00 PM, 10 nonsmokers aged 18 to 29 (mean age 23.8 ± 3.6) years were put on a defined diet low in polycyclic aromatic hydrocarbons during the course of the experiment. The following night and day (control day) any exposure to ETS was avoided. On the first day, the subjects spent 8 h in an unventilated, ordinarily furnished room of 45 m³ in order to simulate exposure conditions. On the second day (exposure day), the subjects were exposed to ETS at a level of approximately 10 $\mu\text{L/L}$ CO in the unventilated room for 8 h. The exposure session started at 8:30 AM and was finished at 5:00 PM with a 30-minute lunch break at noon. The subjects were only allowed to leave the room to go to the lavatory. The smoke was

generated by two smokers smoking cigarettes, so that a CO level of about 10 $\mu\text{L/L}$ was maintained. Blood samples were taken before the subjects entered the room at 8:00 AM and after they had left the room at 5:00 PM on both the control and exposure days. Each subject sampled his 24-h urines on two consecutive days. Sampling began after discarding the first morning urine at approximately 8:00 AM on the control day. The subjects were dismissed from the laboratory on the morning after the exposure day.

Experiment 2. Experiment 2 was carried out in the same way as Experiment 1 except for the following changes: It was performed in two separate runs, each of them comprising 5 nonsmokers and 5 smokers. Six of the nonsmokers had participated in Experiment 1. The age of the subjects ranged from 19 to 28 (mean age 23.7 ± 2.7) and from 24 to 44 (mean age 32.4 ± 7.0) years for the 10 nonsmokers and the 10 smokers, respectively. The smokers had to refrain from smoking after admission to the laboratory until entering the exposure room on the exposure day. After this they were free to smoke cigarettes of their own brand. The CO level on the exposure day of Experiment 2 varied between 20 and 25 $\mu\text{L/L}$. The 10 smokers served as positive controls for the biological monitoring.

Room monitoring

The air sampling tubes were installed in breathing height of a sitting person at the end of the room, which was opposite to where the smokers sat. Carbon monoxide and nitrogen oxides were measured continuously by a Carbon-Monoxide Analyzer, Model 8310 (Monitor Labs Inc., USA) and a Nitrogen-Oxide Analyzer, Model 8840 (Monitor Labs Inc., USA), respectively. Nicotine was sampled on Extrelut-filled tubes for 0.5 to 2 h (flow rate 2.4 L/min). The loaded tube was alkalized by ammonia, and the alkaloid was eluted with 20 mL ethyl acetate. In the dried and concentrated eluate, nicotine was determined by capillary gas chromatography (Klus et al. 1987). Formaldehyde was measured according to the method of Kennedy and Hill (1982). The aldehyde was derivatized with N-benzylethanolamine to form N-benzylloxazolidine that was detected by capillary gas chromatography.

Biomonitoring

Carboxyhemoglobin (COHb) was measured by means of a CO-Oximeter, Model 182 (Instrumentation Laboratories Ltd., USA) immediately after drawing the blood sample. Cotinine in serum and urine was detected by a radioimmunoassay as described by Langone

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et al. (1973) and modified by Haley et al. (1983). Thioethers in urine were determined by measuring sulfhydryl groups with Ellman's reagent after alkaline hydrolysis of the thioether bonds. Corrections were made for free sulfhydryl compounds (Van Doorn et al. 1979; Heinonen et al. 1983).

Extraction of urine samples for mutagenicity testing was performed according to the original method of Yamasaki and Ames (1977) modified by Mohtashamipur et al. (1985). Briefly, aliquots of 400 mL filtered urine samples were adjusted to pH 8.0 (NaOH) and loaded on an XAD-2 column (0.7 x 8 cm). After washing with 3 mL of water, the column was extracted with 40 mL methanol. The first drops were discarded until the brownish eluate appeared. The eluate was evaporated to dryness under reduced pressure at 65 to 70°C. The residue was dissolved in 2 mL DMSO. This procedure is reported to lower the histidine concentration in the extract to undetectable levels (Mohtashamipur et al. 1985). The urine concentrates were tested for mutagenicity by the Salmonella (TA98) microsome (S9-mix derived from Aroclor-treated rats) assay. Each sample was determined in triplicate using 10, 25, 50 and 75 μ L urine concentrate, corresponding to 2, 5, 10 and 15 mL of original urine. The slope of the linear part of the dose-response curve obtained by linear regression technique was used to calculate the mutagenic activity in the 24-h urine. In each case, the steepest of the three slopes was used for further analyses.

Statistical analysis

The one sample t-test for differences (exposed minus nonexposed) was applied.

RESULTS

Room monitoring

In both experiments the ventilation conditions were rather poor, which is reflected by high room temperatures (27 to 30°C), high relative humidities (70 to 90%) and an unpleasant odor. The time patterns of CO during the control and exposure days of both experiments are shown in Fig. 1. The distribution of cigarettes smoked by time in both experiments is indicated at the bottom of Fig. 1. During Experiment 1, a total number of 42 cigarettes was smoked. During each session of Experiment 2, the total number of cigarettes smoked amounted to 100.

The CO levels hardly exceeded 2 μ L/L on the control day. During the exposure day of Experiment 1, the CO concentration fluctuated around 10 μ L/L. For Experiment 2, the CO level was between 20 and

25 μ L/L and reached 27 μ L/L at the end of the session. The CO concentration decreased in the 30 min lunch break during which smoking was stopped and the room was kept closed.

Approximately the same time pattern was observed for NO. Control-day levels amounted to 30 nL/L, whereas the NO concentrations increased approximately to 160 nL/L in Experiment 1 and to 300 nL/L during Experiment 2. The NO₂ levels measured during the exposure day of Experiment 1 hardly exceeded the control-day levels amounting to 10 nL/L and were even lower in the afternoon. Interestingly, the peak concentration was observed during lunch. The reasons for this are not known yet. During the exposure day of Experiment 2, the NO₂ concentration amounted to about 150 nL/L and showed a slight increase during the course of the session. The NO₂ level in Experiment 2 is more than proportionately elevated when compared to Experiment 1.

The formaldehyde level, which was 15 to 20 μ g/m³ on the control day, rose to about 40 μ g/m³ during Experiment 1 and approximated 60 μ g/m³ in the afternoon of Experiment 2. The formaldehyde concentrations are only weakly associated with the number of cigarettes smoked in the test room.

The nicotine concentration increased from a background level of 15 to 20 μ g/m³ up to 100 μ g/m³ during the first experiment and up to 180 μ g/m³ during the second. It is worth mentioning that the nicotine level follows more strictly the number of cigarettes smoked than the other ETS components.

Biomonitoring

The results of the biomonitoring are summarized in Table 1. The COHb remained constant in nonsmokers on both control days and increased by 0.7% on the average after the first exposure regimen and by 2.0% after the second exposure regimen. For smokers, the COHb decreased to nonsmoker levels during the phase of smoking abstinence and increased by 7.0% after smoking cigarettes for 8 h. On the average, the smokers smoked 14.7 \pm 7.8 (range 5 to 34) cigarettes in the 8-h exposure period and 8.8 \pm 4.3 (4 to 15) cigarettes during the following evening.

The ETS-related increase in serum cotinine amounted to about 1 ng/mL after exposure in Experiment 1 and to about 5 ng/mL in Experiment 2. Due to the long half-life of cotinine, the smokers' serum cotinine levels remained more than 100 times higher than those of the nonsmokers. The cotinine concentrations measured in smokers during the control day and before the start of smoking on the exposure day reflect the well-known slow cotinine elimination kinetics.

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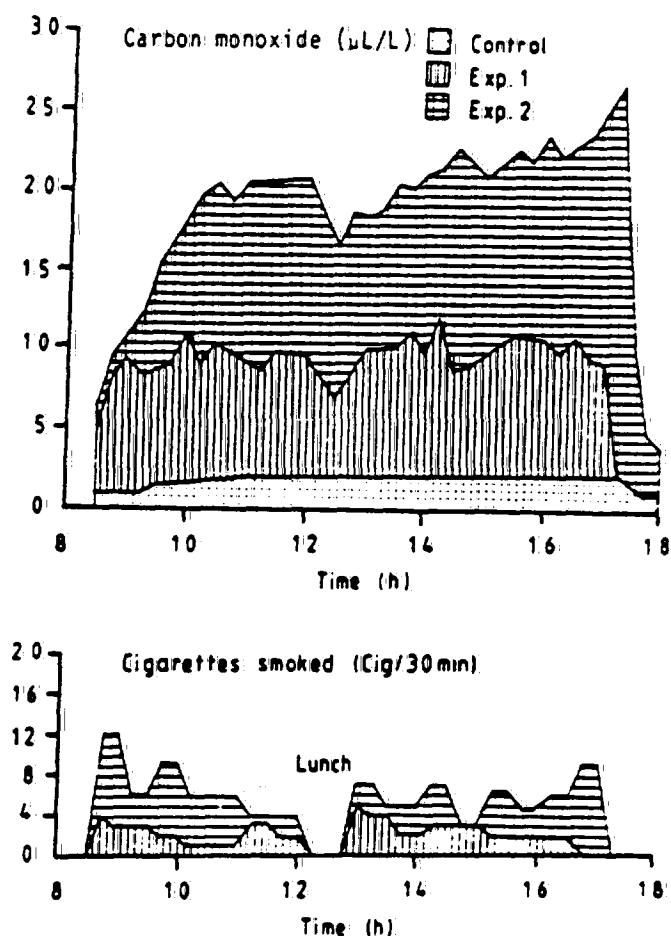


Fig.1. Indoor air concentration of carbon monoxide and number of cigarettes smoked during Experiment 1 and Experiment 2.

Table 1. COHb and cotinine in serum and urine of ETS-exposed nonsmokers and cigarette smokers. Means (Standard deviation).

	Experiment 1		Experiment 2			
	Nonsmokers (N=10)		Nonsmokers (N=10)		Smokers (N=10)	
	Control day	Exposure day	Control day	Exposure day	No smoking	Smoking
COHb (%)						
8.00 a.m.	0.34 (0.15)	0.18 (0.02)	0.63 (0.19)	0.65 (0.32)	2.44 (1.10)	0.81 (0.44)
5.00 p.m.	0.32 (0.13)	0.87 (0.04)***	0.62 (0.13)	2.69 (0.13)***	1.24 (0.40)***	7.87 (2.20)***
Serum cotinine (ng/mL)						
8.00 a.m.	0 (0)	0 (0)	1.2 (1.7)	0.4 (1.0)	378 (130)	146 (51)
5.00 p.m.	0 (0)	1.1 (0.3)***	0.9 (1.6)	4.9 (0.9)***	242 (84)	264 (84)***
Urine cotinine (ug/24h)						
	0 (0)	23 (8)**	21 (13)	67 (26)***	4490 (1800)	3580 (1280)*

Statistical comparisons: COHb and serum cotinine: 5.00 pm vs 8.00 am; urine cotinine: exposure vs control day.
Levels of significance: * : $p < 0.05$; ** : $p < 0.01$; *** : $p < 0.001$

Basically, the same pattern is to be seen for the urinary cotinine excretion. The nonsmokers excreted on the average 15 $\mu\text{g}/24\text{ h}$ (Experiment 1) and 46 $\mu\text{g}/24\text{ h}$ (Experiment 2) more cotinine than on the corresponding control days.

After ETS exposure in Experiment 1, the thioether excretion was elevated by 13.9 $\mu\text{mol}/24\text{ h}$ ($p=0.07$) and in Experiment 2 by 21.4 $\mu\text{mol}/24\text{ h}$ ($p<0.01$) (Fig. 2). The average amount of thioethers excreted by the 10 smokers on the control day was still elevated (89.1 $\mu\text{mol}/24\text{ h}$) as compared with nonsmokers and increased to 136.1 $\mu\text{mol}/24\text{ h}$ ($p<0.01$) after smoking (Fig. 2).

Urinary mutagenicity shows a high interindividual variability under all experimental conditions (Fig. 3). In nonsmokers, no increase was found in Experiment 1, whereas a slight but statistically not significant increase ($p=0.15$) was observed in Experiment 2 (Fig. 3). After smoking, the mutagenic activity of the urine was significantly increased ($p<0.01$) (Fig. 3).

DISCUSSION

The indoor air data reveal that the exposure level in Experiment 1 may correspond to real-life situations (Klus et al. 1987; Triebig and Zober 1984; Sterling et al. 1982), although the daily exposure time is usually less than 8 h; on average, it is about 3 h (Letzel and Johnson 1984; Ministerium für Arbeit, Gesundheit und Soziales NRW, 1987). The exposure level and duration in Experiment 2 bears no relation to a real-life situation. These considerations are also supported by the COHb measurements. An increase by 0.7% in COHb as observed after ETS exposure in Experiment 1 is rarely reached by nonsmokers in normal indoor environments where smoking is permitted (Szadkowski et al. 1976; Jarvis et al. 1983). The cotinine levels in serum and urine as measured in the experiments are not suitable for comparison with results of field studies on ETS exposure (Jarvis et al. 1983; Wald et al. 1984). This is due to the protocol of the study, which includes only a single

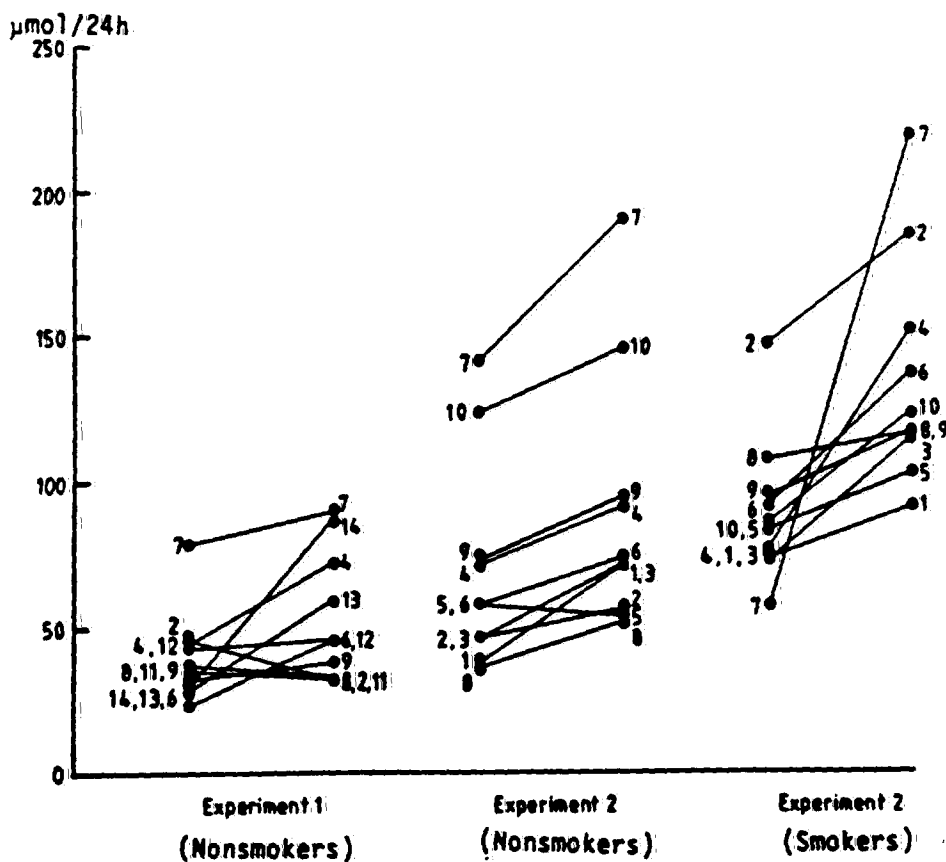


Fig. 2. Individual changes of urinary thioether excretion after ETS exposure and cigarette smoking. (The figures refer to individual subjects.)

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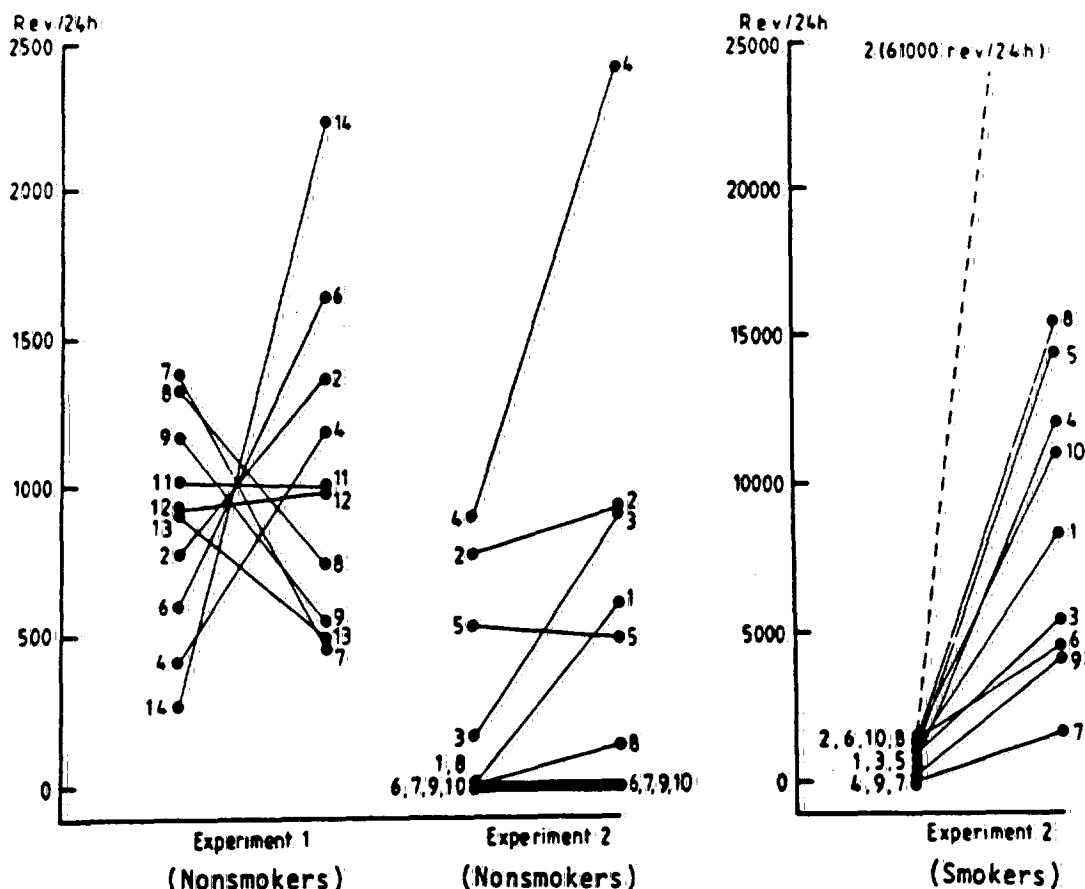


Fig.3. Individual changes of urinary urinary mutagenicity after ETS exposure and cigarette smoking. (The figures refer to individual subjects.)

ETS exposure period of 8 h and to the relatively long half-life of cotinine of about 20 h. Both facts prevent cotinine from attaining steady-state levels in the subjects.

While an increase in thioether excretion after smoking is well documented (Van Doorn et al. 1979), an increase in thioether excretion after high ETS exposure has been described for the first time. Recently, Sorsa et al. (1985) were unable to find a change in thioether excretion after moderate ETS exposure (4 $\mu\text{L/L}$ CO, 5 h/d, for 2 days) of 6 volunteers in a chamber experiment. The relatively low exposure level, insufficient dietary control, and the fact that habitual smokers were used in the study might explain the discrepancy with the present study. According to the findings, smoking does not lead to a substantially higher increase in thioether excretion when compared to ETS exposure. The smokers, however, show higher base levels in thioether excretion, which could be due to their previous smoking and/or dietary factors. The latter might also be the reason for the somewhat elevated thioether level in the nonsmokers

on the control day of Experiment 2. Preliminary data obtained in the laboratory show that smokers have to refrain from smoking and be kept under controlled dietary conditions for at least 3 to 5 days before a steady state in the daily amounts of thioethers excreted is attained.

The absence of a significant increase in urinary mutagenicity after ETS exposure as found in this study is in accordance with results reported by Sorsa et al. (1985) and Husgafvel-Pursiainen et al. (1987), but at variance with those published by Bos et al. (1983) and Mohtashamipour et al. (1987). The increase in mutation rates as found by Bos et al. (1983) was just significant and amounted to about 4% of that measured in smokers, an increase that is rather similar to the rise by 2% that was observed in Experiment 2. Mohtashamipour et al. (1987) reported an increase in urinary mutagenicity after experimental ETS exposure that is comparable to smoking 4 or 5 cigarettes actively and that would correspond to an ETS-related rise in urinary mutagenicity of 15% relative to that found in smokers of 30 cig/d. This is clearly in con-

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trast to the findings of Bos et al. (1983) and the present study's results. Differences in the urine extraction methods might have contributed to this discrepancy. Mohtashamipur et al. (1987) speculate that the urine volume tested (15 mL in the present experiment vs. 50 mL in their study) might have caused this discrepancy. While this might play a role in this study, it is not relevant to the study of Bos et al. (1983), who also tested urine aliquots of about 50 mL. On the other hand, increasing cytotoxicity that interferes with the Ames test when testing urine volumes considerably higher than 15 mL was observed. With the exception of the study by Husgafvel-Pursiainen (1986) from work-related exposure to ETS, all studies, including this one, were performed under experimental conditions. The time-integrated ETS exposure doses are estimated to be similar or somewhat lower in the studies of Bos et al. (1983) and Mohtashamipur et al. (1987), while they are substantially lower in the study of Sorsa et al. (1985) when compared to Experiment 2. Sorsa et al. (1985) reported a nonsignificant elevation of mutagenic activity after ETS exposure that amounts to 70% of that found after smoking, despite the relatively low ETS exposure level. This discrepancy could partly be attributed to the low mutation rates found after smoking and partly to the fact that abstinent smokers (instead of nonsmokers) were used for this experiment. Except for smokers when smoking, it was found that the number of revertants induced by the urine extracts were low and usually less than twice the spontaneous mutation rate. Additionally, no dose-response relationship was observed with urine concentrations of ETS-exposed nonsmokers. This indicates that the criteria of a positive test result as established by Yamasaki and Ames (1977) were not met. Furthermore, the high variability in the urinary mutagenicity, which was observed despite strictly controlled conditions, suggests that yet unknown methodological and endogenous factors clearly outweigh marginal increases in urinary mutagenicity after ETS exposure. Moreover, the relevance of an increased urinary mutagenicity with respect to cancer risk is unclear as of yet. Rüdiger and Lehnert (1988) stated that an increased urinary mutagenicity does not necessarily indicate a higher exposure with genotoxic substances, since the compounds active in the Ames test may be formed from inactive urinary precursors by the in vitro metabolizing system. On the other hand, a negative Ames test does not exclude a genotoxic burden of the organism, since active metabolites may react with cellular constituents in the body and are thus not excreted in the urine. These considerations lead to

the conclusion that measuring the urinary mutagenic activity, at least in passive smokers, is not an appropriate method of predicting an increased risk to human health.

Another aspect of the mutagenicity data attracted interest: the relationship of urinary excretion of thioethers and mutagenicity. The intracellular glutathione-S-alkyl-transferase/glutathione system efficiently protects the organism from destructive effects of electrophilic substances. Thioethers (mercapturic acids) are excreted in the urine as final products of this detoxifying pathway (Chasseaud 1979). The mutagenic activity in urine as detected by the *S. typhimurium* microsome assay may at least partly be caused by electrophilic intermediates or their precursors that escape from reactions in the organism and appear in the urine. Van Doorn et al. (1979) observed rather parallel time courses of urinary thioethers and mutagenicity in smokers who were advised to stop smoking and to start again. No measurable increase in urinary mutagenicity after extreme ETS exposure, despite a significant increase in thioether excretion, was seen. On the other hand, both parameters are significantly increased after smoking. The data are compatible with the assumption that either the mutagenicity in smokers' urines may be caused by compounds that ETS-exposed nonsmokers do not take up in measurable amounts or that the cellular glutathione-S-alkyl-transferase/glutathione system in nonsmokers may detoxify substances that otherwise would reach the urine and could be detected as mutagens by the Ames test.

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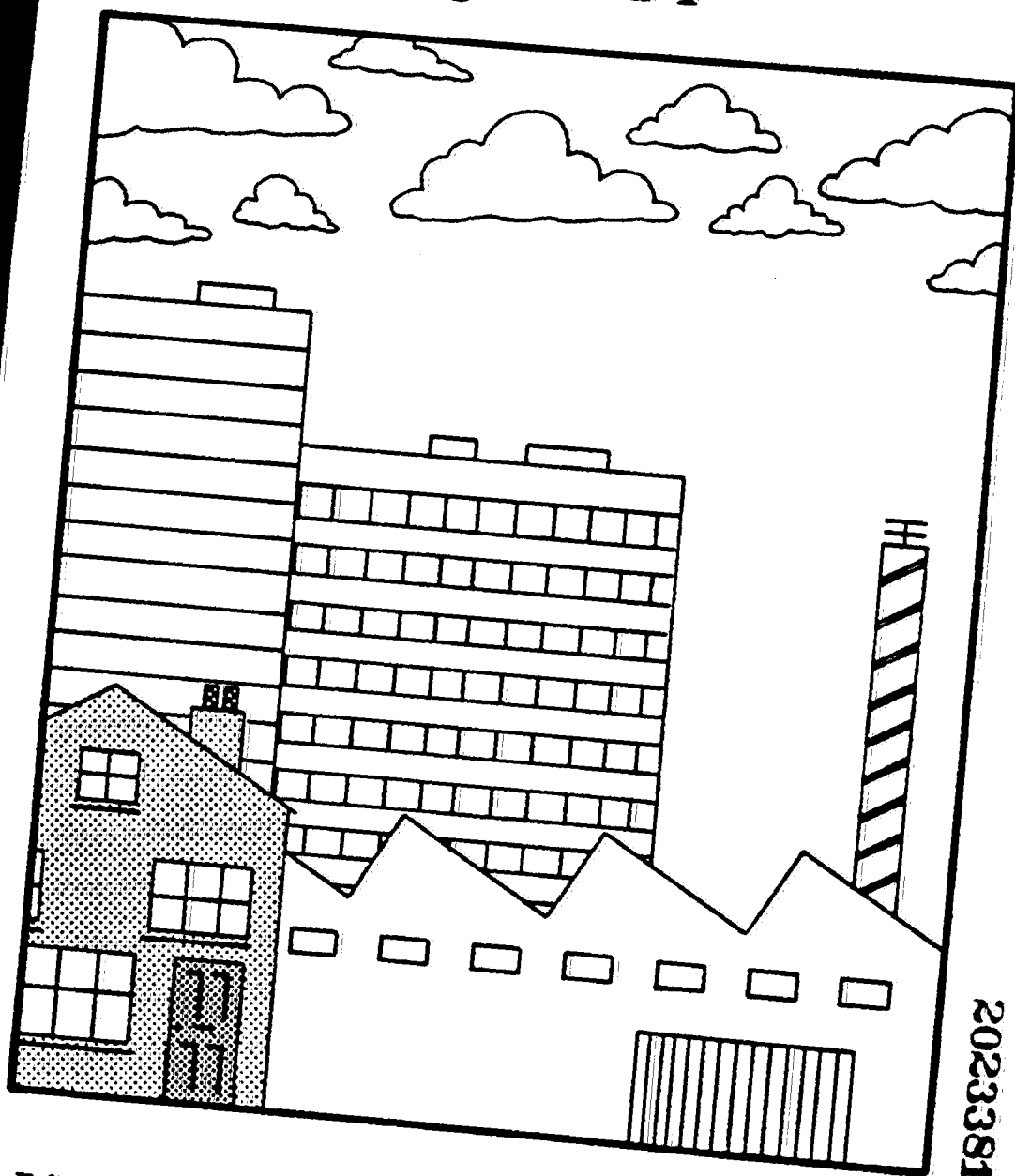
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INDOOR AND AMBIENT AIR QUALITY



Edited by R. Perry
and P.W. Kirk



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QUANTITATIVE AND QUALITATIVE DIFFERENCES IN TOBACCO SMOKE UPTAKE BETWEEN ACTIVE AND PASSIVE SMOKING

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ABSTRACT

Although mainstream smoke (MS) and sidestream smoke (SS) of cigarettes contain the same substances, they are released in different amounts and, moreover, the ratio (SS/MS) varies from substance to substance. Environmental tobacco smoke (ETS) to which the passive smoker is exposed consists for the most part of highly diluted SS and to a lesser degree of exhaled MS. In view of the differences in the physicochemical properties (particle size distribution, pH) between MS and ETS, and due to the fact that smokers differ in their inhalation pattern from passive smokers, the amount of tobacco smoke compounds taken up from ETS cannot be calculated by means of a simple linear extrapolation based on the active uptake of smokers. In order to obtain some insight into this mechanism, we measured the levels of COHb and the urinary excretion of cotinine, thioethers and mutagenicity after controlled ETS-exposure and following cigarette smoking. The results lead us to conclude that doses taken up by smoking and passive smoking cannot be compared on the basis of one single biomarker and that the urinary mutagenicity in smokers may be caused by compounds which ETS-exposed non-smokers do not take up in effective amounts.

INTRODUCTION

For obvious reasons, the possible health risk of passive smoking is often linearly extrapolated from the values found in smokers. For instance, Russell (1) calculated the number of deaths due to passive smoking in the UK to be about 1000 per year by comparing salivary cotinine levels in non-smokers and smokers. These extrapolations, whatever the reality of their basis might be, imply that ETS (mainly consisting of SS) which is breathed in by passive smokers and MS which is inhaled by smokers, have the same biological properties and that they both reach and are deposited in the same areas of the lung. Theoretically, one would have to assume that very low doses evoke the same biological responses as higher doses. For a number of reasons, however, a simple linear risk extrapolation from active to passive smoking is questionable. 1) The SS/MS ratios of cigarettes are substance related and range from 0.5 to 100 (2). 2) SS consists of smaller particles (3) and has a

higher pH (2) compared to MS. 3) Deposition rate of breathed SS in the human respiratory tract was found to be 11 % (4), whereas smoking results in deposition rates of 50-90 % (5). 4) ETS is more or less aged before it is breathed in by the non-smoker, whereas the smoker inhales fresh MS. In vitro investigations show that the former is less toxic than the latter (6).

From this it can be concluded that the passive smoker takes in very low doses of a complex mixture of substances which differs from MS in physical, chemical and biological terms. Additionally, low doses of toxic and genotoxic substances can be more effectively eliminated by defence systems such as the mucociliary clearing mechanisms and the glutathione-S-transferase/glutathione (GST/GSH) system than is the case with higher doses. In order to gain a greater insight into what happens to potentially genotoxic compounds after active and passive smoking we performed two controlled studies during which the urinary excretion of thioethers and mutagens was measured in addition to other tobacco smoke-related dosimetric parameters.

MATERIAL AND METHODS

Study 1 (passive smoking)

The study has been described in more detail elsewhere (7). Briefly, 10 non-smokers were exposed for 8 hours to ETS at levels equivalent to 10 and 25 ppm CO in two separate experiments. Each experiment included a control day during which an 8 hour sham-exposure took place. Blood was collected before and after the stay in the experimental room. On both the control and exposure days, 24 h-urine was sampled. Carboxyhemoglobin was measured in fresh blood by means of a CO-oximeter (IL 182, Instrumentation Laboratories Ltd.). Cotinine (8), thioethers (9) and mutagenicity (10) in urine were determined using slightly modified published methods. All biochemical data were collected under blind conditions.

Study 2 (active smoking)

Eight male smokers (mean age: 31.3, range: 25-42 years) stopped smoking 3 days before admission to the laboratory. The subjects stayed in the laboratory from the eve of the first experimental day until the morning after the eighth day. During this period the volunteers received a controlled diet low in polycyclic aromatic hydrocarbons. The meals were identical in quality and quantity on each of the 8 days of the study. On day 1 and 2 the subjects did not smoke. On day 3, 4, 5, 6, 7, and 8 they smoked 1/2, 1, 2, 4, 8, and 16 cigarettes of their own brand, respectively. The interval between lighting two cigarettes was standardized to 30 min. Smoking ceased on each day at 4 pm. Blood samples for COHb measurements were drawn every day immediately before and after smoking. Urine sampling periods throughout the study were from 8 am to 4 pm, from 4 pm to midnight and from midnight to 8 pm. In each urine fraction cotinine, thioethers and mutagenicity were determined. In order to compare the results with those of study 1, 24-hour urinary excretion rates (8 am to 8 am) were calculated.

RESULTS AND DISCUSSION

The results of both studies are summarized in Table 1. For the ETS-exposed non-smokers, dose-related increases in COHb, urinary cotinine and thioether excretion were observed, this was not the case for urinary mutagenicity.

Table 1: Dose-related increases in COHb and urinary excretion of cotinine, thioethers and mutagenicity (TA98 + S9 mix) after controlled active and passive smoking

		M e a n (S.D.)			
		Δ COHb (%)	Cotinine (μg/24h)	Thioethers (μMol/24h)	Mutagenicity (Rev/24h)
Study 1 (N=10)					
<u>Passive smoking</u>					
Experiment 1	Control	0(0.1)	8(8)	40.0(15.4)	875(371)
	ETS exp. (10 ppm CO)	0.7(0.1)	23(8)	53.9(22.8)	1069(565)
Experiment 2	Control	0(0.1)	21(13)	69.3(36.3)	236(358)
	ETS exp. (25 ppm CO)	2.1(0.2)	67(26)	90.7(44.8)	548(757)
Study 2 (N=8)					
<u>Active smoking</u>					
day	Cig/d				
1	0	-	113(74)	98.3(19.4)	1424(893)
2	0	0(0.1)	32(18)	97.9(20.7)	1171(864)
3	0.5	0.5(0.2)	77(23)	89.5(23.7)	808(533)
4	1	0.9(0.3)	116(39)	82.4(19.3)	1615(519)
5	2	2.0(0.8)	227(93)	72.2(22.4)	1845(839)
6	4	3.1(0.9)	316(106)	84.8(19.0)	1713(591)
7	8	5.1(1.0)	858(304)	93.8(18.5)	3051(714)
8	16	7.3(1.8)	1626(729)	103.7(25.6)	5645(3589)

The increases in COHb after both exposure regimes exceed those reported in real-life situations (11, 12). The amounts of cotinine excreted in the urine of our subjects should not be compared with data obtained in field observation, since cotinine has a half-life of about 20 hours and thus does not attain steady-state concentrations in our study. The excretion of thioethers is regarded as an indicator of exposure to electrophilic (and therefore potentially mutagenic and/or carcinogenic) substances (13). We found an increase of thioether excretion in both ETS exposure experiments, which was statistically significant in the high dose experiment (25 ppm CO). To our knowledge, this is the first report of a significantly elevated thioether excretion after ETS exposure. (14). We relate this finding to the strictly controlled dietary conditions in our study minimizing confounding effects of food.

The urinary mutagenicity after ETS exposure was found to be at the detection limit of the Ames-test. When applying the criteria of a positive test result (doubling of the spontaneous mutation rate and linear dose-relationship (10) there was no indication of mutagenicity in the urine of ETS-exposed non-smokers. The absence of a significant increase in urinary mutagenicity after ETS exposure found by us is in accordance with the results of two other studies (14, 15), but at variance with those by Bos et al. (16) and Mohtashamipour et al. (17). Bos et al. (16) reported an increase after passive smoking amounting to 4 % of the values measured after smoking. This is similar to our findings (2 %). However, Mohtashamipour (17) reported a much higher ETS-related increase equivalent to the mutagenicity found after smoking 4-5 cigarettes.

An interesting result of Study 1 is the difference in the two urinary parameters, the relatively high thioether excretion, but the lack of a clear increase in mutagenicity. The increase in thioether excretion amounted to 30-40 % compared with values measured after active smoking. This increase clearly exceeds the relative increases in COHb and urinary cotinine. Two hypotheses might explain this finding: 1) In ETS-exposed non-smokers the cellular GST/GSH system may detoxify substances which otherwise would have reached the urine and could be detected as mutagens by the Ames-test, while in smokers the capacity of this system may be overloaded, 2) the mutagenicity in smokers' urine may be caused by compounds which ETS-exposed non-smokers do not take up in measurable amounts.

In order to distinguish between these two possibilities we performed a controlled smoking experiment during which smokers smoked increasing amounts of cigarettes, starting with a very low dose (1/2 cig/d). If the first hypothesis is correct, the amount of thioethers excreted should rise at lower doses before urinary mutagenicity increases. As can be seen from Table 1, measurable increases in urinary thioethers and mutagens were only observed when 4 or more cigarettes/d were smoked. With respect to mutagenicity, this result confirms observations of other authors (18). This finding does not confirm hypothesis 1.

Thioether excretion declined during the first 5 days of the study (0 to 2 cig/d). This could be due to the introduction of a controlled diet and the cessation of smoking. Since the subjects abstained from smoking for 5 days and received the controlled diet for 2 1/2 days before resuming smoking the possibility of a slow component of the thioether elimination mechanism has to be considered. As shown in Figure 1, this assumption is also supported by the excretion of mutagens and thioethers in the individual 8 h urine fractions. A circadian rhythm for the thioether excretion was observed in nearly all of our subjects. Excretion levels peaked in the samples taken between 4 pm and midnight. These high levels do not appear to be related to smoking and may be attributable to the diet. Furthermore, smoking seems to affect the thioether excretion less rapidly and less effectively than the excretion of mutagens (Table 1, Figure 1). The highest urinary mutagenicity recorded was observed within 8 or 16 hours after smoking at least 4 cigarettes. This finding is in line with Kado et al. (19) who reported an elimination half-life of approximately 7 h. Since there is no absolute complementary relationship between urinary thioethers and

mutagenicity, we conclude that the second hypothesis is the most probable.

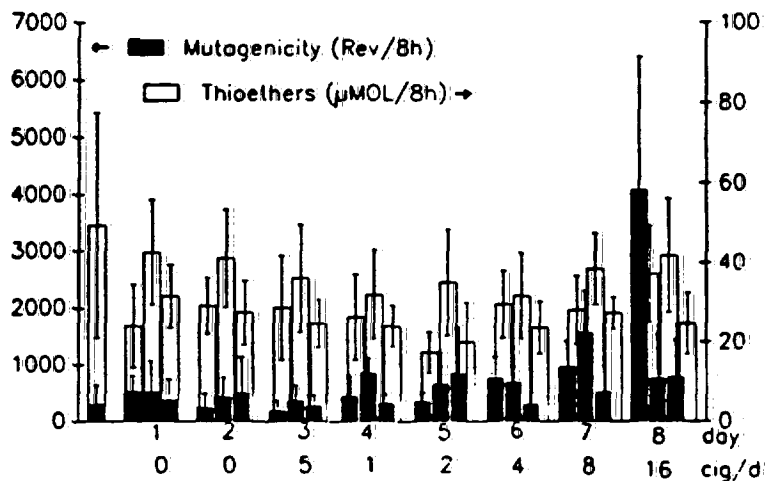


Figure 1b

Average amount (with bars for standard deviation) of mutagenicity and thioethers excreted in the 8 h-urine fractions. For each day the sampling periods were: 8 am - 4 pm, 4 pm - midnight and midnight - 8 am. The two bars on the left side of the graph represent data of the 8 h-urine fraction (midnight - 8 am) before the start of the experiment.

A regression analysis was performed on the data of study 2, considering only the linear part of the dose-response-relationships. The slopes (increase/cig) were related to the increases measured after ETS exposure (Table 2).

Table 2: ETS exposure dose expressed as "cigarette equivalents" by comparing data of Study 1 and Study 2

	Δ CORb	Cotinine	Thioethers	Mutagenicity
ETS exposure (10 ppm CO)	1.55	0.15	6.82	(0.59)
ETS exposure (25 ppm CO)	4.63	0.46	10.64	(0.95)

The extent of ETS exposure expressed as "cigarette equivalent" clearly depends on the biological parameter used, indicating that "cigarette equivalents" based on single markers are not a valid measure of dose for ETS exposure. This result confirms the

theoretical considerations made in the INTRODUCTION and emphasizes the difference in quality between ETS and MS exposure. The surprisingly high increase in thioether excretion after ETS exposure needs further investigation.

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Urinary Mutagenicity, Hydroxyphenanthrene, and Thioether Excretion After Exposure to Environmental Tobacco Smoke

G. Scherer, K. Westphal, and F. Adlkofer

Summary

In two controlled studies 10 non-smokers each were exposed for 8 h to environmental tobacco smoke (ETS) equivalent to 10 ppm CO (Experiment 1) and 25 ppm CO (Experiment 2). During the control and the exposure period the room air was monitored for CO, NO, NO₂, nicotine and formaldehyde. Biomonitoring included determination of carboxyhemoglobin (COHb), cotinine in serum and urine, and urinary excretion of five different monohydroxyphenanthrenes (OH-PHE), thioethers and mutagenic activity (as detected by the Salmonella typhimurium TA 98/microsome assay). The observed increases in COHb indicate that ETS exposure in Experiment 1 was substantially higher than in a real-life situation, whereas that in Experiment 2 bore no relation to common passive smoking. This is mainly due to the fact that high ETS exposure in a real-life situation is usually much shorter than 8 h per day. Urinary excretion of OH-PHE and mutagenicity was not significantly increased after both experimental ETS exposures. In contrast to this, excretion of thioethers was elevated after ETS exposure in Experiment 1 ($P = 0.07$) and Experiment 2 ($P < 0.001$). Our results suggest that non-smokers in real-life situations take up very low doses of ETS constituents, which in case of potentially genotoxic substances are likely to be detoxified.

Introduction

There has been much controversy in the scientific literature about the health risk to non-smokers due to passive smoking [8]. Since epidemiology which provides most of the evidence for a positive correlation between ETS exposure and chronic diseases is extremely sensitive to bias and confounding factors in low risk associations [24], solid risk assessments should not be based on epidemiological data alone. Toxicological data have to be considered as well in order to come to firm conclusions. Dosimetry of ETS exposure is clearly a prerequisite for risk evaluations. Nicotine, cotinine and thiocyanate in body fluids as well as COHb are normally used as biochemical markers for ETS exposure [10]. While in fact these markers permit comparing tobacco smoke exposure during active smoking with that during passive smoking to some limited extent, they do not allow direct estimates of the non-smoker's body burden by toxic and, in particular, genotoxic substances. This is due to the quantitatively different composition of mainstream smoke (inhaled by the smoker) and sidestream smoke (breathed in highly diluted form by the non-smoker) [12], the different aging time-related toxicities of both smoke types [21], and the different patterns of inhalation between active and passive smoking resulting in different deposition rates and distributions in the respiratory tract [6, 19].

In order to get some more insight into the exposure doses of potentially genotoxic substances after ETS exposure, we extended the biomonitoring in two controlled ETS exposure experiments to the excretion of five different monohydroxyphenanthrenes (used as marker for exposure to polycyclic aromatic hydrocarbons (PAH)), the excretion of thioethers (used as a marker for exposure to electrophilic substances), and the excretion of mutagens in the urine.

Material and Methods

Subjects

Twenty-four healthy male subjects (14 non-smokers and 10 smokers) aged 18 to 44 years (mean age 22.0 years) volunteered to take part in either one or both experiments. After admission to the laboratory on Friday evening they completed a questionnaire on socio-economic and life-style factors as well as on their ETS exposure during the last 48 h.

Protocol

Experiment 1: After admission to the laboratory at 8 pm, 10 non-smokers aged 18 to 29 (mean 23.8 ± 3.6) years were put on a defined diet low in polycyclic aromatic hydrocarbons during the course of the experiment. The following night and day (control day) any exposure to ETS was avoided. On the first day the subjects spent 8 h in an unventilated, ordinarily furnished room of 45 m³, in order to simulate exposure conditions. On the second day (exposure day) the subjects were exposed to ETS at a level of approx. 10 ppm CO in the unventilated room for 8 h. The exposure session started at 8.30 am and was finished at 5 pm with a 30-min lunch break at noon. The subjects were only allowed to leave the room to go to the lavatory. The smoke was generated by two smokers smoking cigarettes, so that a CO level of about 10 ppm was maintained. Blood samples were taken before the subjects entered the room at 8 am and after they had left the room at 5 pm on both the control and exposure day. Each subject sampled his 24 h urines on two consecutive days. Sampling began after discarding the first morning urine at approximately 8 am on the control day. The subjects were dismissed from the laboratory on the morning after the exposure day.

Experiment 2 was carried out in the same way as experiment 1 except for the following changes: It was performed in two separate runs each of them comprising five non-smokers and five smokers. Six of the non-smokers had participated in experiment 1. The age of the subjects ranged from 19 to 28 (mean 23.7 ± 2.7) and from 24 to 44 (mean 32.4 ± 7.0) years for the 10 non-smokers and the 10 smokers, respectively. The smokers had to refrain from smoking after admission to the laboratory until entering the exposure room on the exposure day. After this they were free to smoke cigarettes of their own brand. The CO level on the exposure day varied between 20 and 25 ppm. The 10 smokers served as positive controls for the biological monitoring.

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Table 2. Biomonitoring in ETS exposed non-smokers and cigarette smokers. (Mean \pm SD)

	Experiment 1		Experiment 2			
	Non-smokers (n = 10)		Non-smokers (n = 10)		Smokers (n = 10)	
	Control day	Exposure day	Control day	Exposure day	Control day (no smoking)	Exposure day (smoking)
COHb (%)						
before	0.34 \pm 0.15	0.18 \pm 0.02	0.63 \pm 0.19	0.65 \pm 0.32	2.44 \pm 1.10	0.81 \pm 0.44
after	0.32 \pm 0.13	0.87 \pm 0.04	0.62 \pm 0.13	2.69 \pm 0.13	1.24 \pm 0.40	7.87 \pm 2.20
Serum cotinine (ng/ml)						
before	0 \pm 0	0 \pm 0	1.2 \pm 1.7	0.4 \pm 1.0	377.8 \pm 129.7	145.7 \pm 51.0
after	0 \pm 0	1.1 \pm 0.3	0.9 \pm 1.6	4.9 \pm 0.9	242.3 \pm 84.3	244.0 \pm 84.4
Cotinine in urine (μ g/24 h)	8 \pm 8	23 \pm 8	21 \pm 13	67 \pm 26	4,485 \pm 1,795	3,584 \pm 1,278
		..			.	
Σ Hydroxyphenanthrenes in urine (μ g/24 h)	5.63 \pm 2.38	5.33 \pm 2.05	4.60 \pm 0.30	4.28 \pm 0.80	4.82 \pm 0.48	5.55 \pm 0.44
		NS			(NS) ^a	
Thioethers (μ Mol/24 h)	40.0 \pm 15.4	53.9 \pm 22.8	69.3 \pm 36.3	90.7 \pm 44.8	89.1 \pm 24.8	136.1 \pm 38.9
		NS			..	
Mutagenicity Rev./plate ^d	6 - 12	3 - 16	0 - 10	0 - 19	0 - 23	15 - 376
Rev./24 h	875 \pm 371	1,069 \pm 565	236 \pm 358	548 \pm 757	927 \pm 510	13,819 \pm 17,224
		NS			NS	

Levels of significance are as follows: NS = not significant, $p > 0.05$; * $p < 0.05$; ** $p < 0.01$; *** $p < 0.001$.

^a Range of highest mean rates (minus spontaneous rate) of triplicate measurements observed after applying 10, 25, 50 and 75 μ l urine concentrate (corresponding to 2, 5, 10 and 15 ml urine).

^b Two pooled urine samples of five subjects each.

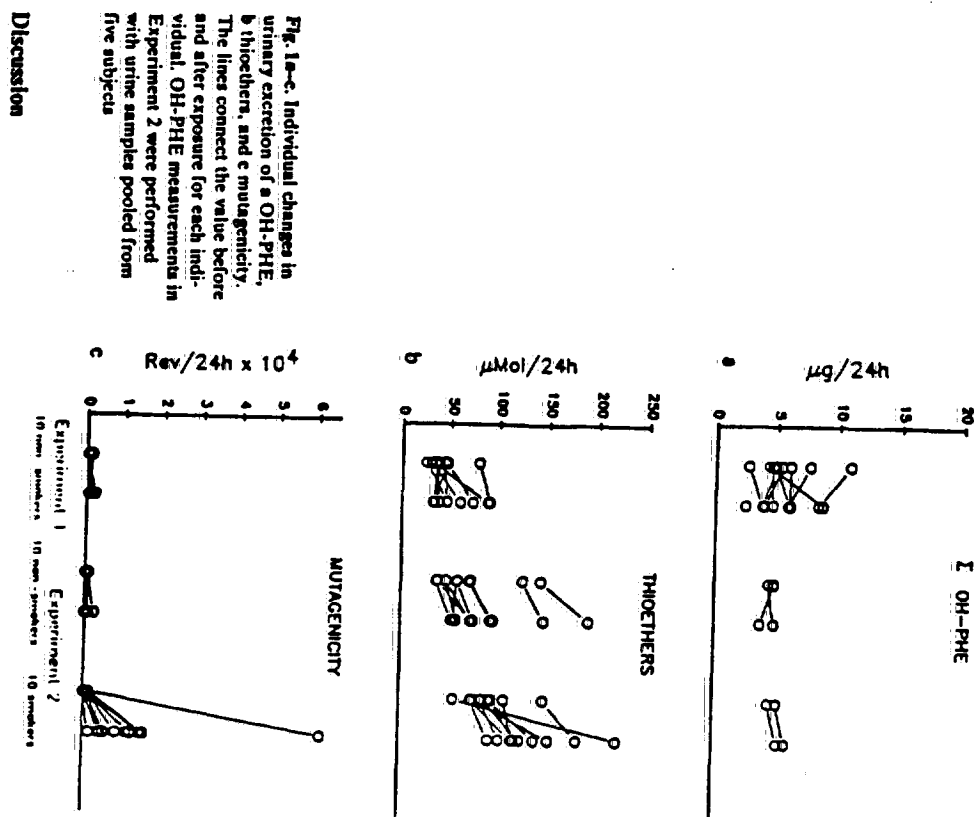


Fig. 1a-c. Individual changes in urinary excretion of a OH-PHE, b thioethers, and c mutagenicity. The lines connect the value before and after exposure for each individual. OH-PHE measurements in Experiment 2 were performed with urine samples pooled from five subjects.

Discussion

Our indoor air measurements reveal that the concentration of ETS in Experiment 1 may correspond to real-life situations [13]. However, average daily exposure time is reported to be usually less than 8 h [15, 16], indicating that exposure conditions in Experiment 1 are higher than commonly achieved. This is confirmed by the observed increases in COHb levels in our Experiment 1 which were found to be 0.7% on the average. Field measurements of COHb in non-smokers working in ETS-polluted rooms result in much lower, if any, increases in COHb [9, 23]. Significant increases in serum and urine cotinine levels were found after ETS exposure in both experiments. However, since steady-state levels were not attained for cotinine in a single 8-h exposure regimen, our data cannot be compared with cotinine concentrations measured under field conditions with repeated exposures.

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The Effects of Environmental Tobacco Smoke on Pulmonary Function

M.D. Lebowitz and J.J. Quackenboss

Summary

A community study in Tucson is underway to evaluate the effects of environmental tobacco smoke (ETS) exposure on acute and chronic pulmonary function, including bronchial reactivity. Study families (about 700) are part of a multi-stage stratified cluster sample. Monitoring for PM₁₀ and PM_{2.5} in the houses showed a good correlation between amount of tobacco smoking in the home and measurements of concentration; distributions of PM by amount smoked do overlap somewhat. Further, PM₁₀ and PM_{2.5} have a very close correlation ($R^2 = 85\%$).

In the first 400 subjects, some relationships have been found between proportions with significant diurnal peak flow (PEF) and ETS (by amount smoked in the home), controlling for PM₁₀ or PM_{2.5} ($\mu\text{g}/\text{m}^3$). These relations of PEF - bronchial lability in association with PM and ETS, some of which are definitely paradoxical, have to be explored further. So far, the acute changes are not reflected in any differences in chronic symptomatology. Further, the acute symptoms have not been associated with ETS-PM; one exception is non-specific, complaint symptoms, which are higher when both ETS and PM₁₀ are highest ($p = 0.08$). It is possible that PEF changes have a similar mechanism. To study the reliability and validity of such relationships, atmospheric nicotine and serum cotinine should be evaluated.

Environmental tobacco smoke has various chemical constituents that are either annoying, irritating, or biologically active (US Surgeon General 1975, 1984). Thus, there are different effects on the primary organ site, the lung (Stein and Weinbaum 1986): annoyance responses affect the lung only in susceptible individuals who may respond with increased airway resistance; a few asthmatics are like this. Irritants can produce several responses, that are dose-dependent. Constituent may be biologically active: they may attract defense and inflammatory cells which may lyse to release proteases, endotoxins, and chemotactic factors, and stimulate the production of mediators, leading potentially to inflammation (and bronchial reactivity), stimulate mucus glands, and/or affect immune responses. Certain constituents may alter DNA or RNA within cells. Irritant responses may be greater in previously sensitized individuals. Biological activity will depend on host status and susceptibility (physiologically, biochemically, immunologically). Biological assays/markers help determine status/susceptibility.

Sensitivity/susceptibility is a function of age as well, and early exposures and infections are quite important (Lebowitz and Burrows 1986). Some of the effects mentioned can be measured by pulmonary function responses, in individuals characterized by their susceptibility or sensitivity (Lebowitz et al. 1987). Acute and chronic effects, and the relationships between them, need to be considered.

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URINARY EXCRETION OF HYDROXY-PHENANTHRENES AFTER INTAKE OF POLYCYCLIC AROMATIC HYDROCARBONS

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EI 87-475 (Received 5 November 1987; Accepted 5 May 1989)

The intake of polycyclic aromatic hydrocarbons (PAH) from various sources including smoking, passive smoking, diet, and workplace was studied. Up to five different monohydroxy-phenanthrenes (OH-PHE) excreted into the urine were used as biomarkers for PAH exposure. The OH-PHE excretion of smokers was slightly but not significantly elevated as compared to nonsmokers. No difference in urinary OH-PHE excretion was observed in nonsmokers before and after exposure to environmental tobacco smoke (ETS) even if the exposure levels were unrealistically high. A diet containing elevated levels of PAH leads to a rise in urinary OH-PHE excretion. A considerable increase in OH-PHE excretion was found in road paving workers as well as in one wood creosoting worker. The amount of OH-PHE excreted was up to 200 times higher than in nonsmoking or smoking controls.

INTRODUCTION

Exposure to polycyclic aromatic hydrocarbons (PAH) has drawn much attention because PAH are considered to be potential human carcinogens (International Agency for Research on Cancer 1983; 1984a; 1984b; 1985; Deutsche Forschungsgemeinschaft 1987). PAH are formed from incomplete combustion or pyrolysis of organic material. They occur in food, water, tobacco smoke, and in polluted air, particularly at some workplaces. PAH from combustion sources or petroleum products form a complex mixture in which phenanthrene is an important quantitative constituent. The disposition kinetics of phenanthrene which is generally found to be nonmutagenic and noncarcinogenic (International Agency for Research on Cancer 1983) in mammals is not known.

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At present, no commonly used biomarker for PAH exposure has been established. Becher and Bjoerseth (1983) proposed the determination of urinary PAH metabolites after chemical reduction to the parent compounds. Jongeneelen et al. (1986) preferred measuring 1-hydroxypyrene in the urine of exposed subjects. Sims (1962) reported excretion of 1-, 2-, 3-, 4-, and 9-hydroxyphenanthrene (free and as sulfuric ester) in urine of rats and rabbits treated with phenanthrene. The same monohydroxy-metabolites (except for 9-hydroxyphenanthrene) were found by Jacob et al. (1982) in vitro experiments with induced rat liver microsomes. The major metabolites of phenanthrene, however, are the 1-, 2-, 3-, 4-, and particularly the 9,10-dihydrodiols (Jacob et al. 1982) which are excreted as various conjugates in the urine (Sims 1962; Horning et al. 1987).

The objective of our investigation was to compare the urinary excretion of monohydroxy-phenanthrenes

(OH-PHE) in subjects exposed to different PAH sources including smoking, ETS, diet, and two different workplaces. For this purpose we determined the urinary excretion of 1-, 2-, 3-, 4-, and 9-hydroxyphenanthrene (see also Hoepfner et al. 1987).

SUBJECTS AND METHODS

Experiment 1 (Smoking)

Six healthy nonsmokers aged 25 to 43 years and six healthy smokers (average daily consumption 25 to 44 cigarettes) aged 23 to 30 years collected a 24 h urine in their normal environments. The subjects completed standard questionnaires on eating, drinking, and smoking habits and life-style. The urine samples were analyzed for OH-PHE.

Experiment 2 (ETS exposure)

Ten nonsmokers were exposed to ETS equivalent to 10 and 20 to 25 $\mu\text{L/L}$ CO under strictly controlled conditions (Scherer et al. 1987). The high ETS concentration was generated by 10 smokers who were also included in the investigation. They smoked between 15 and 25 cigarettes during the exposure period. OH-PHE were measured in each 24 h urine collected during the experiment with 10 $\mu\text{L/L}$ CO. In the experiment with higher ETS exposure we decided

on a single determination of a pooled urine sample from five smokers and five nonsmokers each to reduce the total number of determinations and, of course, the costs of the experiments.

Experiment 3 (Diet)

After admission to the laboratory, eight healthy nonsmokers aged 20 to 38 years were put on a controlled diet low in PAH (potatoes, carrots, and beef) for two days, and on a diet rich in PAH (charcoal broiled meat, leafy vegetables, darkly roasted toast) for an additional two days. Throughout the four days, 24 h urines were collected for OH-PHE determination.

Experiment 4 (Workplace)

The samples were obtained from subjects studied by Jongeneelen (1987), who investigated PAH exposure by measuring 1-hydroxypyrene in the workers' urine. Spot urine samples from seven road paving workers and one wood creosoting worker were analyzed for OH-PHE. In brief, occupational exposure conditions and urine sampling procedures were as follows. For road paving: Hot blends of bitumen (140°C) were sprayed onto an existing road surface and covered by an excess of stone chips (chip sealing). The spraying vehicle was closely followed by a

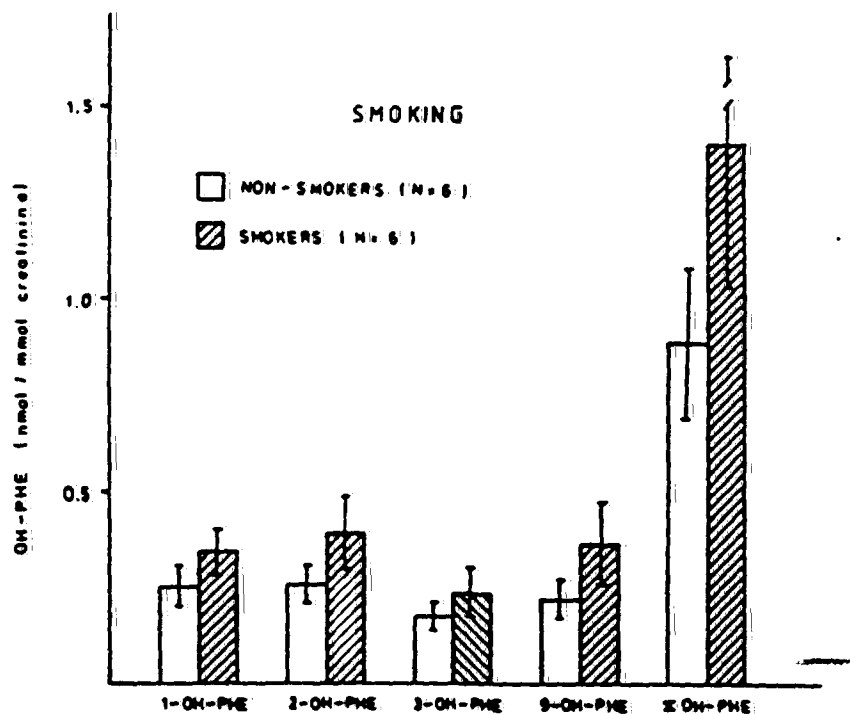


Fig. 1. Urinary excretion of OH-PHE in six nonsmokers and six smokers under real-life conditions ($M \pm SD$).

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chipper vehicle. The subjects included in this study worked close to the described sites. Only pre-work urine samples were available for two workers, and only post-work samples were available for another three workers for OH-PHE analysis. For wood creosoting: One worker at a small wood preserving plant collected pre- and post-work spot urine samples during one week (Tuesday to Monday). From Tuesday to Friday wood was creosoted four times daily. On the following Monday five loads of wood were creosoted.

Analytical methods

Carbon monoxide, nitrogen oxides, formaldehyde, and nicotine in the room air during ETS exposure (experiment 2) were determined as described elsewhere (Scherer et al. 1987).

OH-PHE were extracted from urine by toluene after acid hydrolysis (2 N HCl, 2 h at boiling temperature) and chromatographed on Sephadex LH 20. OH-PHE were quantified by gas chromatography (Perkin Elmer, Model Sigma 2B, fused silica column 25 m x 0.32 mm, SE 54; temperature program: 110 to 160°C at 10°C/min, followed by 160 to 280°C at 3°C/min and constant temperature at 280°C; helium flowrate: 30 cm/s; detector: FID; integrator: Spectra Physics). Details are described elsewhere (Grimmer and Dettbarn 1987). Phenanthrene was extracted from homogenized food samples with cyclohexane after hydrolysis with methanolic KOH (10% KOH). Extracts were filtered through silica gel to remove polar substances. After Sephadex LH 20 chromatography, phenanthrene was determined by gas chromatography under the same conditions as described for OH-PHE (Grimmer and Böhne 1979).

Creatinine in urine was determined photometrically as picrate according to the Jaffe method.

RESULTS

In the field study (experiment 1), smokers show slightly elevated OH-PHE excretions when compared to nonsmokers (Fig. 1). Neither for the single OH-PHE metabolites nor for the sum of these metabolites is the difference between the two groups statistically significant. A similar result was obtained for the smokers in experiment 2 (Fig. 2c).

In the two exposure studies (experiment 2), the ETS concentration was equivalent to 10 $\mu\text{L/L}$ CO and 20 to 25 $\mu\text{L/L}$ CO, respectively; the latter was unrealistically high. The exposure levels are reflected by indoor air concentrations of NO, NO₂, formaldehyde, and nicotine which amounted to 160 nL/L, 20 nL/L, 40 $\mu\text{g/m}^3$, and 60 $\mu\text{g/m}^3$, respectively, in the 10 $\mu\text{L/L}$ CO

exposure regimen as compared to 310 nL/L, 150 nL/L, 50 $\mu\text{g/m}^3$, and 120 $\mu\text{g/m}^3$, respectively, in the 25 $\mu\text{L/L}$ CO exposure regimen. Carboxy hemoglobin increased by 0.7% in the "low" ETS exposure study and by 2.0% in the "high" ETS exposure study (for details see Scherer et al. 1987). Under both experimental conditions no change in OH-PHE excretion was observed (Fig. 2a and b). The two diets used in

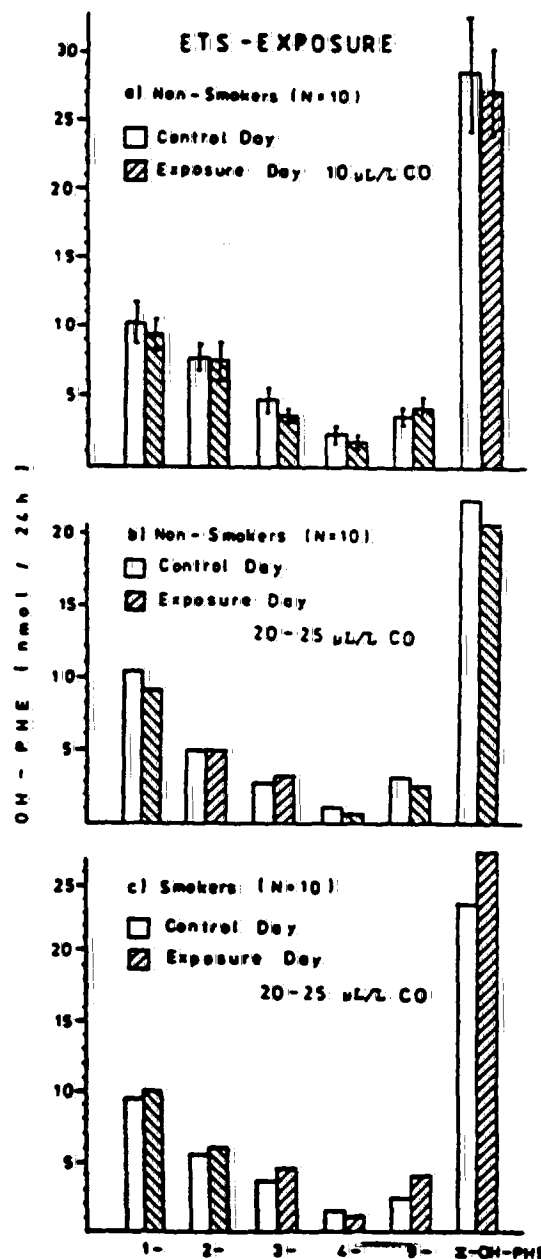


Fig. 2. Urinary excretion of OH-PHE in nonsmokers and smokers after controlled exposure to ETS (a) $M \pm SD$; b) and c) mean of two determinations comprising pooled urine samples from five subjects each).

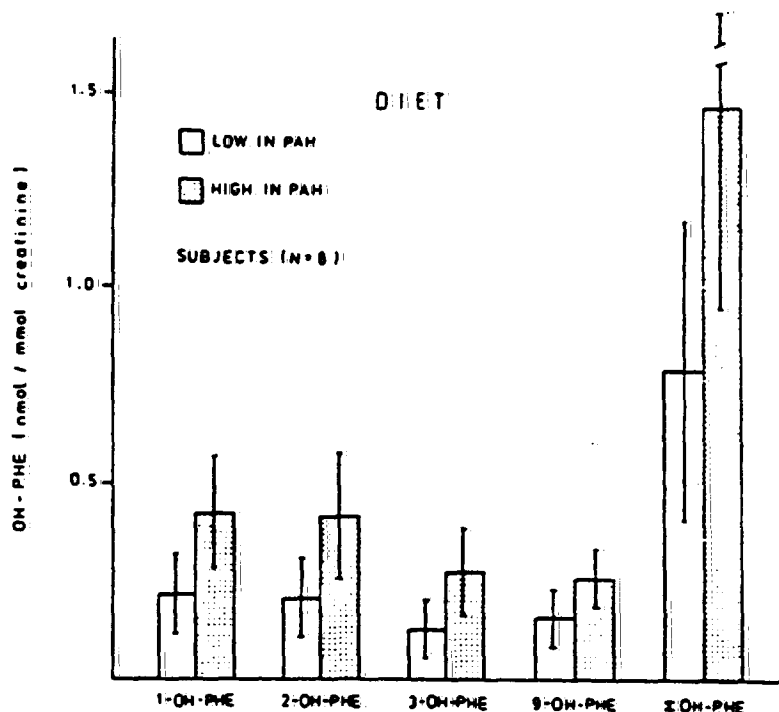


Fig. 3. Urinary excretion of OH-PHE in nonsmoking subjects on a diet differing in PAH content ($M \pm SD$).

experiment 3 differed significantly in PAH content. This is indicated by the phenanthrene concentration which was found to be 0.5 and 4.4 $\mu\text{g}/\text{kg}$ for the low and high PAH diet, respectively. The subjects on the diet high in PAH excreted slightly more OH-PHE when compared to those on the diet low in PAH (Fig. 3). This difference is of borderline significance ($p = 0.10$).

Road paving and wood creosoting workers showed considerably higher amounts of OH-PHE in their urine when compared to the non-occupationally exposed subjects. The pre-work levels of $\Sigma\text{OH-PHE}$ were up to 15 times higher, whereas the post-work levels were up to 200 times higher than those of the subjects in experiment 1, 2, and 3 (Fig. 4). In any case, the highest levels of OH-PHE in urine were always found to be post-shift. As for the creosote worker, only the pre- and post-shift values measured on Monday are shown; the values obtained during the week were even higher.

DISCUSSION

In our first experiment, we found a slight but insignificant increase in urinary OH-PHE excre-

tion after smoking. This finding is in agreement with that of Jongeneelen et al. (1985) who used 1-hydroxypyrene, and that of Venier et al. (1985) who used the reduction method for the determination of OH-PHE as a biomarker for PAH exposure. In contrast to this, Becher and Bjoerseth (1983) reported significantly elevated excretion of phenanthrene and other PAH in smokers as compared to nonsmokers when applying the reduction method. However, the same working group (Haugen et al. 1986; Becher and Bjoerseth 1983) found no significant difference in PAH excretion between occupationally exposed nonsmokers and smokers.

In our second experiment, no measurable increase in urinary OH-PHE excretion was observed after ETS exposure. Based on the estimates in Table 1, the phenanthrene intake through ETS exposure would be about 1/50 of that through smoking. Obviously the increase in urinary OH-PHE is beyond the sensitivity of our biomonitoring even under extremely high ETS exposure conditions (20 to 25 $\mu\text{L}/\text{L}$ CO for 8 h).

The observed elevation in urinary OH-PHE after a diet rich in PAH (experiment 3) is also in line with

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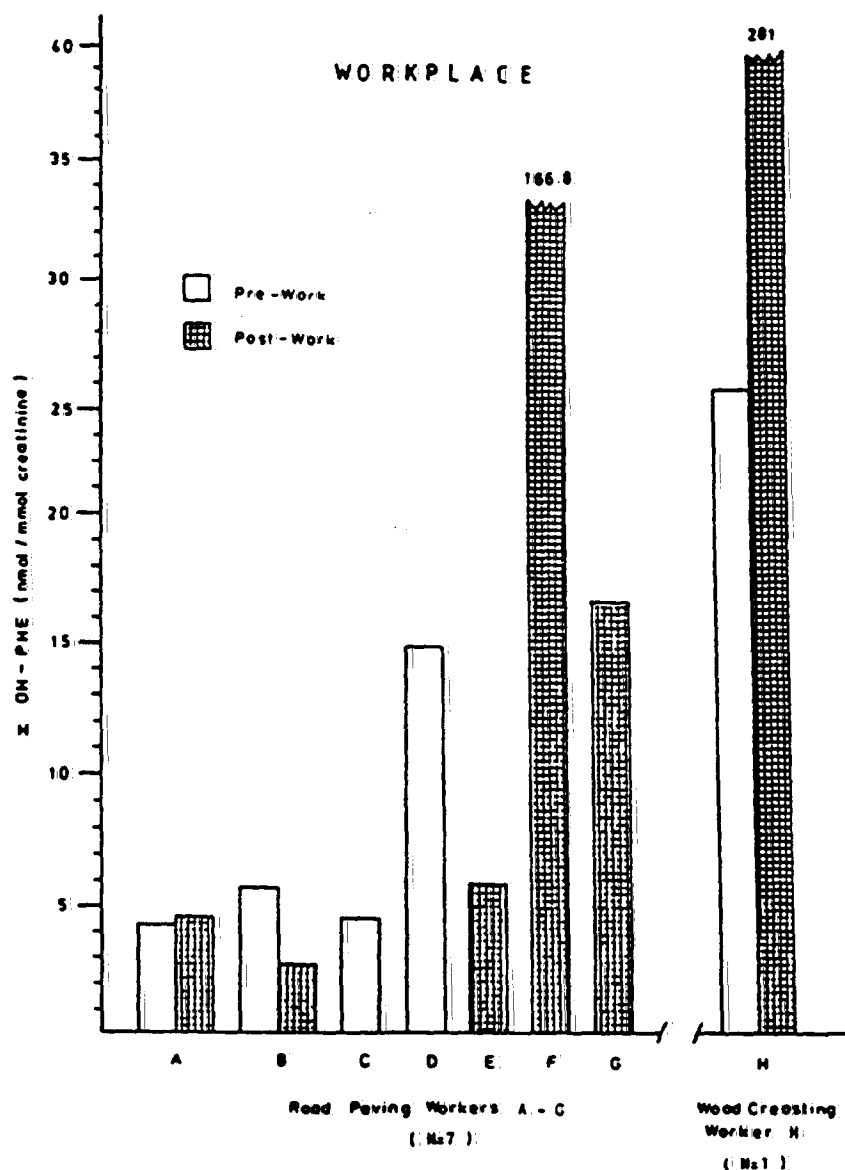


Fig. 4. Urinary excretion of OH-PHE in seven (A-G) road paving workers and in one (H) wood creosoting worker before and after work shift ($M \pm SD$).

the phenanthrene intake as estimated in Table 1. The increase was somewhat higher than that after smoking, but still statistically not significant which is probably due to the small number of subjects investigated. Clearly elevated OH-PHE values were found in subjects exposed to high PAH concentrations at the workplace. Both the road paving workers and the creosoting worker exhibit up to 15 times higher pre-shift OH-PHE values in urine when compared to the occupationally nonexposed subjects. Post-shift values were up to 200 times higher. This is in line with the findings of Jongeneelen (1987) who reported in-

creased post-work urinary 1-hydroxypyrene excretions in the same workers.

The high pre-shift values of OH-PHE observed even after work-free weekends indicate a slow terminal elimination rate of these compounds in phenanthrene-exposed workers. In contrast to this, the initial elimination phase is much faster, lasting only about 12 hours, as can be seen from the pre-shift/post-shift fluctuations on consecutive days. These observations suggest that phenanthrene and pyrene may accumulate in the body of daily exposed subjects.

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Table 1. Estimated daily intake of phenanthrene from different sources. The intake from urban air, ETS, and workplace is calculated based on a respiratory volume of 0.5 m³/h.

Source	Concentration	Intake
Urban air ^{a)}	2 ng/m ³	0.008 µg/8 h
ETS ^{b)}	10 ng/m ³	0.04 µg/8 h
Smoking ^{c)}	100 ng/cig	2 µg/20 cig
Workplace ^{d) e)}	2-90 µg/m ³	8-360 µg/8 h
Diet ^{f)}	-	1.2-10.6 µg

a) Matsumoto and Kashimoto (1985)

b) Husgafvel-Pursiainen et al. (1986)

c) Grimmer et al. (1987)

d) Haugen et al. (1986)

e) Bjoereth et al. (1978)

f) This study; average daily food intake: 2.4 kg

In occupationally nonexposed persons the daily intake of phenanthrene from different sources may reach up to 10 µg (Table 1). The amounts of EOH-PHE in the 24 h urine of these subjects range between 1.2 and 6 µg (Figs. 1 to 3). A discrepancy between estimated daily intake and measured daily excretion of phenanthrene is obvious. Considering the fact that only a small fraction of phenanthrene is renally cleared as OH-PHE (Grimmer and Deubarn 1987), the amount excreted in the urine appears much too high as compared with the amount taken in. The accumulation of this compound as discussed above might at least partly explain this discrepancy. Yet unknown sources of PAH exposure and analytical problems must be considered as well.

In conclusion, our data show that ETS exposure does not lead to a measurable increase in urinary OH-PHE excretion, even if the ETS concentration is extraordinarily high and the exposure time unrealistically long. On the other hand smoking and a diet containing elevated levels of PAH appears to cause slight increases in OH-PHE excretion. A considerable increase in OH-PHE excretion is observed in workers with high occupational PAH exposure. These findings agree with estimated phenanthrene exposure levels from different sources.

We do not know to what percentage the investigated metabolites are formed from the parent PAH and to what extent they are renally cleared. These limitations must be considered when interpreting the results.

Acknowledgments — The authors wish to thank Dr. P. J. Jongeneelen and his co-workers; University of Nijmegen, The Netherlands, for

kindly supplying urine samples from occupationally exposed subjects and for fruitful discussion; and Mrs. Claire Hruby and Mrs. Ursel Stoltz for their expert assistance in preparing the manuscript.

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HYDROXY-PHENANTHRENES IN THE URINE OF NON-SMOKERS AND SMOKERS*

(Environmental tobacco smoke; diet; PAH; urinary mutagenicity)

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(Received 3 September 1986)

(Revision received 23 September 1986)

(Accepted 25 September 1986)

SUMMARY

Urinary hydroxy-phenanthrene (HO-PHE) excretion in non-smokers exposed to environmental tobacco smoke (ETS) is not increased. There is no significant difference in HO-PHE excretion between smokers (S) and non-smokers (NS), though excretion seems to be slightly elevated in smokers. A diet rich in polycyclic aromatic hydrocarbons leads to a rise in urinary HO-PHE excretion as compared to a diet low in polycyclic aromatic hydrocarbons (PAH), coming close to significance. HO-PHE excretion is not correlated with the mutagenic activity in urine.

INTRODUCTION

PAH are thought to be a major cause of lung cancer. In 1984, Becher et al. found a significantly higher PAH excretion in the urine of S as compared to NS, the difference being most evident with regard to PHE [1]. The authors measured various

* Presented at the International Experimental Toxicology Symposium on Passive Smoking, October 23-25, 1986, Essen (F.R.G.).

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Abbreviations: ETS, environmental tobacco smoke; HO-PHE, hydroxy-phenanthrenes; NS, non-smokers; PAH, polycyclic aromatic hydrocarbons; PHE, phenanthrene; S, smokers.

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PAH compounds in urine before and after reduction of the PAH metabolites. The recovery rate for PHE was rather low. In our study the hydroxylated metabolites of PHE were directly determined. In the following we will report on HO-PHE excretion found in NS before and after exposure to ETS, in S, and in subjects on diets differing in PAH contents.

SUBJECTS AND METHODS

Experiment 1

Two groups, each consisting of 5 healthy NS and 5 healthy S were put on a controlled diet. During the first day after admission (control day) the subjects stayed in an experimental room (45 m³, 2 ppm CO, 15–20 µg/m³ nicotine) for 8 h. Smoking was not allowed. On the second day (exposure day) non-smokers of each group were exposed to ETS (100 cigarettes, 22–27 ppm CO, 100–180 µg/m³ nicotine) in the same room again for 8 h. ETS was produced by smokers smoking 100 cigarettes during exposure time. Blood samples were drawn before and after each session for COHb and cotinine determination. 24-h urines were collected for cotinine determination. HO-PHE were measured in pooled 24 h urine aliquots from each group.

Experiment 2

6 healthy NS and 6 healthy S (25–44 cigarettes/day) collected their 24-h urines in their normal environment. For the determination of cotinine, blood samples were drawn from the subjects when returning their urine bottles. Cotinine and HO-PHE were measured in the urine.

Experiment 3

After admission to the laboratory, 8 healthy non-smokers were put on a diet low in PAH for 2 days, and on a diet rich in PAH for a further 2 days. Throughout the 4 days the 24-h urines were collected and analysed for HO-PHE and mutagenic activity.

Methods

CO was continuously recorded on a CO analyzer (Model 8310, Monitor Labs Inc., U.S.A.). Nicotine was analysed by capillary gas chromatography [2]. COHb was quantified using a CO-Oximeter (Model 182, Instrumentation Laboratories Ltd.). Cotinine in serum and urine was measured by radioimmunoassay [3]. HO-PHE in urine were quantified by gas chromatography (Perkin Elmer, Model Sigma 2B, fused silica column 25 m × 0.32 mm, SE 54; temperature programme: 110–160°C/10°C per min, followed by 160–280°C/3°C per min, constant temperature 280°C; helium flow rate: 30 cm/s; integrator: Spectra Physics [4]. *Salmonella typhimurium* was used for urinary mutagenicity testing (TA98 + S9 from Aroclor-treated rats) [5, 6]. Analyses in urine were carried out in coded samples under blind conditions.

RESULTS

The results of Exp. 1 are shown in Table I. On the exposure day a significant rise in COHb and serum cotinine was observed in S and NS, whereas HO-PHE excretion did not vary between control day and exposure day. Essentially, S differed from NS by an elevated cotinine level in serum and in urine. However, no differences were found between S and NS in terms of their urinary HO-PHE excretion.

To check the validity of our results, a second experiment was carried out. The results are summarized in Table II. The excretion of individual HO-PHE metabolites as well as the total excretion of HO-PHE tended to increase in S as against NS. On the other hand, the mean volume of the 24-h urine in S was by about 18% higher than in NS (1335.0 ± 664.8 vs. 1095 ± 377.2). Since there is a significant correlation between urine volume and HO-PHE excretion ($r = 0.7$; $P < 0.01$) the weak increase in HO-PHE excretion in smokers might partly be due to their elevated urinary volume.

In a third experiment we investigated the effect of a diet differing in PAH contents. The HO-PHE excretion as well as the mutagenic activity in urine rose after changing from a low- to a high-PAH diet. The increase in HO-PHE was of borderline significance (Fig. 1). The rises in HO-PHE excretion and mutagenic activity were not correlated.

DISCUSSION

We conclude from our study that the contribution of ETS and even of cigarette smoking to urinary HO-PHE excretion is small, while diet accounts for it to a larger extent. If we assume that HO-PHE in the urine of S is a general indicator of other PAH metabolites, our data are in line with those of Venier et al. [7], but in contrast to those reported by Becher et al. [1]. Michels and Finbrodt [8] come to the same conclusion as that reached by Venier et al. [7] and by us; however, their values for

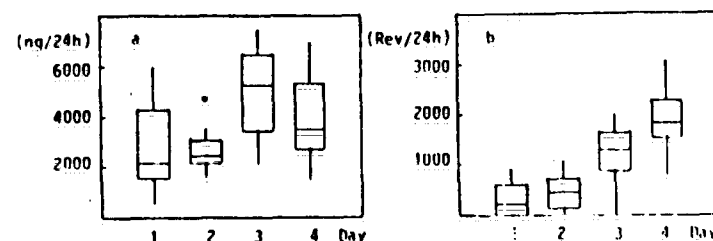


Fig. 1. HO-PHE excretion in 8 subjects on a diet low in PAH (days 1 and 2) and high in PAH (days 3 and 4) (a) and the urinary mutagenicity in the same subjects (b). Data are presented as box plots, for explanation see [11] (Experiment 3).

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COHb, COTININE IN SERUM AND URINARY EXCRETION OF COTININE AND HO-PHENANTHRENES IN NON-SMOKERS AND SMOKERS BEFORE AND AFTER EXPOSURE TO ETS
HO-PHE = 5-HO-PHE, 1-HO-PHE, 3-HO-PHE, 4-HO-PHE, 9-HO-PHE (\pm SD)* (Experiment I)

	Group A				Group B			
	Non-smoker (n=5)		Smoker (n=5)		Non-smoker (n=5)		Smoker (n=5)	
	Control	Exposure	Control	Exposure	Control	Exposure	Control	Exposure
COHb (%)								
8 am	0.4 \pm 0.2	0.9 \pm 0.2	2.9 \pm 0.9	1.1 \pm 0.2	0.6 \pm 0.2	0.4 \pm 0.1	2.0 \pm 1.2	0.5 \pm 0.3
5 pm	0.4 \pm 0.0	2.7 \pm 0.1	1.3 \pm 0.3	8.4 \pm 2.1	0.6 \pm 0.2	2.4 \pm 0.2	1.2 \pm 0.5	7.3 \pm 2.5
Cotinine in serum (ng/ml)								
8 am	1.2 \pm 2.2	0.4 \pm 1.3	396.2 \pm 104.7	157.4 \pm 50.4	1.2 \pm 1.3	0.3 \pm 0.4	359.4 \pm 161.3	134.0 \pm 54.4
5 pm	1.0 \pm 2.2	5.3 \pm 0.8	231.8 \pm 161.3	272.4 \pm 86.7	0.8 \pm 0.8	4.0 \pm 0.9	210.8 \pm 98.9	215.6 \pm 80.6
Cotinine in urine (μ g/24 h)	15.8 \pm 14.6	76.4 \pm 31.7	3250.8 \pm 1886.7	4006.4 \pm 1170.5	25.4 \pm 11.1	58.8 \pm 19.3	3718.4 \pm 1491.7	3162.2 \pm 1363.8
HO-PHE* in urine (μ g/24 h)	4.4	4.8	4.5	5.2	4.8	3.7	5.2	5.9

*HO-PHE were analysed in pooled 24-h urine aliquots.

TABLE II

COTININE IN SERUM AND URINARY EXCRETION OF COTININE AND HO-PHENANTHRENES IN NON-SMOKERS AND SMOKERS (\pm SD) (Experiment 2)

	Cotinine in		1-HO-PHE (μ g/24 h)	2-HO-PHE (μ g/24 h)	3-HO-PHE (μ g/24 h)	9-HO-PHE (μ g/24 h)	Σ HO-PHE (μ g/24 h)
	Serum (ng/ml)						
	Urine (μ g/24 h)	Urine (μ g/24 h)					
NS (n=6)	11.3	21.9 \pm 11.3	0.81 \pm 0.41	0.83 \pm 0.34	0.58 \pm 0.43	0.73 \pm 0.54	2.95 \pm 1.69
S (n=6)	428.6 \pm 188.0	7328.0 \pm 3989.2	11.14 \pm 0.62	1.24 \pm 0.67	0.75 \pm 0.42	1.18 \pm 0.34	4.30 \pm 2.40

urinary PAH excretion appear to be extraordinarily high. In addition, we found mutagenic activity not to be correlated with urinary HO-PHE excretion. Our data support the findings of Venier et al., who did not observe a relationship between urinary PAH excretion and mutagenic activity in urine [7].

According to Husgafvel-Pursiainen et al. [9], the quantity of PHE inhaled during an 8-h day may reach 0.04 μ g (10 ng PHE/m³ \times 4 m³/8 h). A smoker of 20 cigarettes per day may inhale up to 2 μ g PHE [10]. The intake of PHE from the diet may vary from 1.2 to 10.6 μ g per day depending on the PAH content (Hoepfner et al., unpublished data). The daily urinary HO-PHE excretion of up to 6 μ g as found in our study can thus hardly be explained in terms of PHE intake from smoking and diet only. When we come to interpret our findings, still unknown metabolic processes as well as methodological problems have to be discussed.

ACKNOWLEDGEMENTS

The authors wish to thank Dr. G. Henze, Mrs. C. Hruby and Ms. C. Vollert for invaluable assistance in preparing the manuscript.

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An Introduction to the Study of Smoking Using Urinary Hydroxyproline

H. Kasuga

Summary

The indicators used to identify the effects of environmental tobacco smoke (ETS) and exposure to low level nitrogen dioxide (NO_2) on the respiratory system are so weak that they can not be detected using traditional markers such as the increased prevalence of respiratory symptoms and a decrease in lung function. After studying urinary hydroxyproline (HOP) starting in 1977, we first reported the significant relationship between HOP and smoking, ETS and NO_2 in the air, in 1981. Since then, the coherent association between urinary HOP and the established pathological and biological development of lung diseases has been studied. Bias problems based on confounding factors, misclassification of nonsmokers and over- or underestimation of ETS effects also have been discussed.

Among articles presented by us during the past 4 years, several papers and some arguments for and against this study on urinary HOP were introduced:

- 1) The effect of cessation from smoking on the urinary excretion of hydroxyproline [19].
- 2) A prospective repeated cross-sectional study on the possible health effects caused by automobile exhaust and passive smoking [20].
- 3) Impact of smoking on the concentration and activity of alpha-1-antitrypsin in serum, in relation to the urinary excretion of hydroxyproline. Matsuki H, Kasuga H et al., 1988.
- 4) Behavior of urinary hydroxyproline and effect of cigarette smoking in silicosis. Osaka F, Kasuga H, Matsuki H et al., 1985.
- 5) Opinions contrary to the relationship between urinary hydroxyproline and smoking, ETS and NO_2 .

Introduction

Hydroxyproline (HOP) is one of the essential constituents in collagen and elastine and is a unique one which is not found in other tissues. Therefore, urinary HOP is regarded to be a potential candidate for the study of the breakdown of lung tissue due to smoking and environmental tobacco smoke (ETS).

As is generally known, the index symptom such as "a persistent cough and phlegm" based on the BMRC Questionnaire [1] is used frequently as a clinical marker, but it is not applicable for ETS effects because prevailing concentrations of ETS are estimated to be less than 1% of an undiluted mixture of sidestream and second-hand mainstream smoke. Therefore, urinary HOP as a biochemical marker for ETS effects appeared on the stage, and a causal relationship between smoking including ETS and its health effect was

reported by Matsuki et al. [2] in 1981. However, some confounding factors which influence the excretion of HOP into urine have been found. Accordingly, it is very important to control these factors, including growth and age [3], disorders in hormone secretion, various collagenoses, outdoor or indoor air pollution with nitrogen dioxide (NO_2), pregnancy, and abnormalities in collagen metabolism in other sites than the lung. For details on this problem, we refer to our reports [4-9] so far; our method for HOP analysis [10] is recorded in the manual [11] for passive smoking, IARC, WHO, 1987. Our improved method is suitable for routine determination of HOP in urine at concentrations up to at least 400 ng/ml. The limit of detection is about 50 ng/ml. The autoanalyzer can handle 40 samples per hour. It is very difficult to obtain 24-h urine samples from many individuals, but the ratio of hydroxyproline to creatinine in a spot urine sample, particularly collected after fasting, is representative for the quantity of hydroxyproline in 24-h urine. Determination of urinary creatinine is performed routinely.

The amount of HOP excreted in urine is affected by the gelatin content of food, except in case of urine collected after fasting. Even if gelatin is ingested the day before urine sampling, its influence on urine HOP can be avoided by discarding the urine collected in the early morning after a 10-12-h overnight fast and collecting a fasting urine sample 2 h later.

As the first step of the study on smoking effects, it is necessary to classify the selected subjects according to the amount of exposure to cigarette smoke, directly or indirectly. In case of smokers, they can report their own smoking habits in the response to the question by means of interview and questionnaire. But in order to express the personal exposure levels to ETS for non-smokers, we are forced to ask for the smoking habits of potential smokers who supply the ETS. Since such answers are apt to be biased it may cause misclassification of the non-smoking subjects. Therefore, some biochemical markers have been used to assess the exposure to cigarette smoke including ETS. Among them, urinary cotinine has shown to be the most reliable marker for active and passive smoking. But it is not always easy to use this complicated measurement for hundreds or thousands of subjects in epidemiologic surveys. Therefore, a new questionnaire [12] has been developed. The questions in the old questionnaire were revised and enlarged by inserting only a phrase "How many cigarettes did you smoke in the presence of non-smoking spouse and child at home a day?". This new questionnaire showed a significantly high correlation between urinary cotinine levels and the number of cigarettes smoked in the presence of nonsmokers [12] (Table 1).

On the other hand, it may be necessary to use much more detailed questionnaires for studying the relationship between ETS and lung cancer, as Dr. Wynder mentioned. The interview with such a questionnaire is now used in an international survey sponsored by the IARC [13], to provide information on past and current active and passive smoking status. This troublesome questionnaire with as many as 20 sheets may be needed for studying lung cancer because it has been suggested that exposure to cigarette smoke during childhood may cause lung cancer later in life, and its incubation period may be more than ten years.

Since our study using urinary HOP aims at detecting preclinical signs of COLD (chronic obstructive lung diseases) except asthma, it may be possible to abbreviate most of the question sheets. In an actual epidemiological survey, it may be a practical way to examine all the subjects with such an improved questionnaire, and to check for report bias by measuring urinary cotinine. Originally the ETS effect is so weak that we can not always obtain a significant result according to circumstances; e.g. studies carried out by Verplanke et al. [14] in Holland and by Matsuki et al. [15] on Chinese women in Hong Kong were unsuccessful. So, it is necessary to use a sufficient number of subjects on

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Table 1. Correlation coefficients among urinary cotinine level and involuntary smoking. (From [23])

Winter	Child Co/CR	Mot. Co/CR	Fam. Cig/day	Fam. Cig house/day
Child Co/CR	-	0.396*	0.494**	0.626**
Mot. Co/CR	-	-	0.799**	0.820**
Fam. Cig/day	-	-	-	0.851**
Fam. Cig house/day	-	-	-	-
Summer	Child Co/CR	Mot. Co/CR	Fam. Cig/day	Fam. Cig house/day
Child Co/CR	-	0.250	0.389*	0.526**
Mot. Co/CR	-	-	0.426*	0.730**
Fam. Cig/day	-	-	-	0.854**
Fam. Cig house/day	-	-	-	-

* $p < 0.05$, ** $p < 0.01$

Remarks: Child Co/CR: Cotinine to creatinine ratio in children, Mot. Co/CR: Cotinine to creatinine ratio in mothers, Fam. Cig/day: Familial smoking per day at home, Fam. Cig house/day: Familial smoking at home per day in the presence of non-smokers

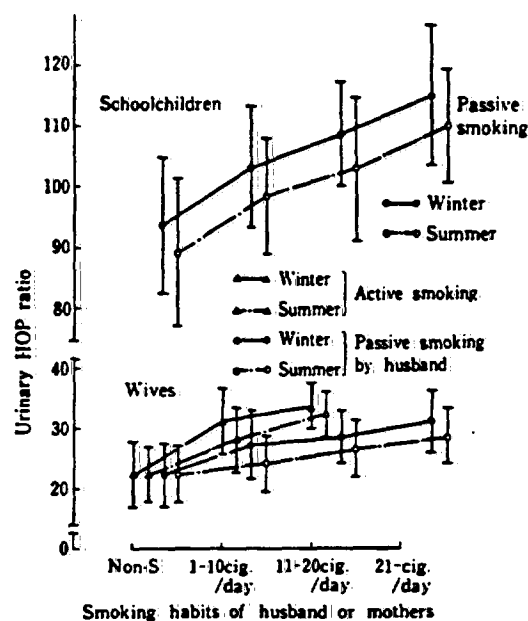


Fig. 1. Seasonal variation of urinary hydroxyproline to creatinine ratio

condition that each bias and confounding factor is under control, and to find a potential internal and external consistent trend by a prospective way, even though each association in the subgroup is weak. Several interventional studies [16-18] with rodents were conducted recently for smoking effects but these ended in failure to show an increase in

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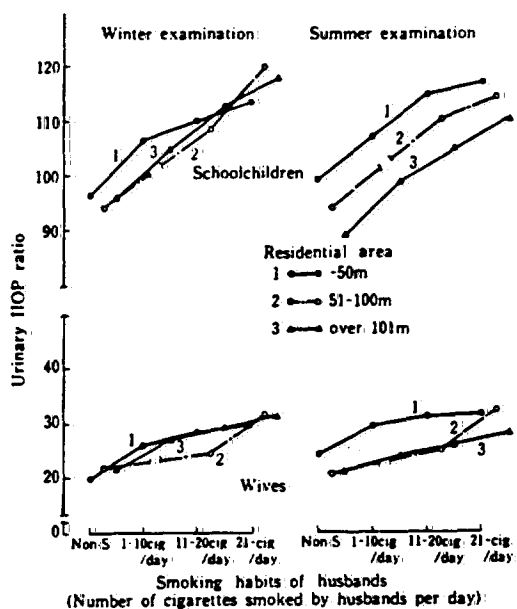


Fig. 2. Passive smoking effects with HOP-ratio by distance from heavy traffic road.

urinary HOP. It seems that a major part of the failure can be ascribed to a defective experimental plan using the short term exposure with low-level ETS. Furthermore, nonsmoking workers are exposed to ETS at offices frequently but measurement of ETS at offices is more difficult than at home. Therefore, at present it may be advisable to select non-smoking, non-working, housewives and schoolchildren as the study subjects. And such an investigation carried out in summer time may be a problem that requires careful consideration because the high level ETS is hard to be formed due to well-ventilation at home in summer [4] (Figs. 1, 2). On the contrary, health effects induced by NO_2 in automobile exhaust should be measured in a summer period because they are masked by higher levels of indoor air pollution which often results from kitchen and space heating in the winter season.

In conclusion, although we have made a gratifying progress in this field, there still remains much to be done. Opinions presented so far may be summarized in some studies conducted during four years after the review [9] on this problem stated at the International Symposium on Effects of Indoor Air Pollution with Special Reference to Nitrogen Oxides and Smoking sponsored by Tokai University and WHO, in 1984, as follows.

The Effect of Cessation from Smoking on the Urinary Excretion of Hydroxyproline [19]

The increased urinary HOP levels due to smoking decrease with time, after discontinuation of smoking, and approached to the lower levels found among non-smokers. This observation must be useful for supporting the positive dose-effect relationship between urinary HOP and smoking. Furthermore, it is important from a public health and

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Fig. 3. HOP-ratio of a smoking cessation clinic group and a fathers group of schoolchildren in the same area; ●—● Smoking cessation clinic group (just after cessation); N: 49; Age: 49.0 ▲ control (non-S) group; N: 6; Age: 30.7 ○—○ fathers group; N: 345, Age: 42.3

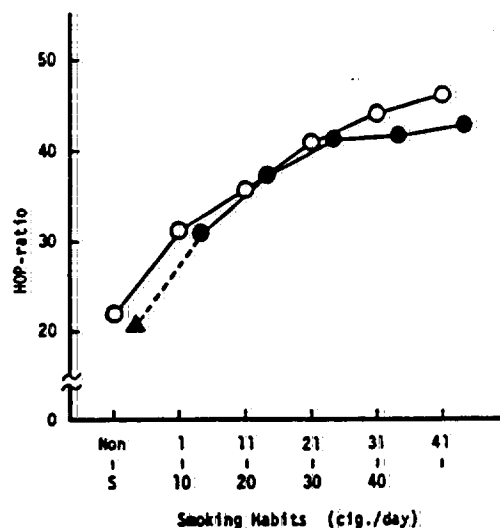
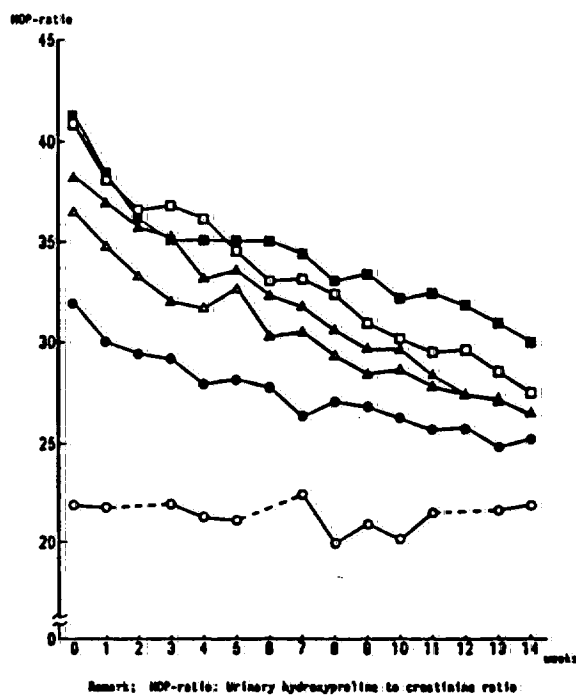


Fig. 4. Arithmetic means of HOP-ratio by amount of cigarette smoking after cessation from smoking; ■—■ 41 cig./day; □—□ 31-40 cig./day; ▲—▲ 21-30 cig./day; △—△ 11-20 cig./day; ●—● 1-10 cig./day; ○—○ non-smokers



Remark: HOP-ratio: Urinary hydroxyproline to creatinine ratio

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Table 2. Calculated proportional and rate constant, correlation and time to reach non-smokers HOP-ratio levels for each group of ex-smokers.

Ex-smoking (cig./day)	k (week)	lnA	r	No.	Time (weeks)
1-10	-0.0751	2.279	0.431**	4	61
11-20	-0.0673	2.630	0.613***	17	73
21-30	-0.0754	2.821	0.611***	11	68
31-40	-0.0709	2.925	0.819***	10	74
41-	-0.0429	2.853	0.391**	7	120

** $p < 0.01$; *** $p < 0.001$.model: $\ln(C_i/C_0) = \ln A + kt_i + e_i$

where C_i is the HOP-ratio of i -th sample, t_i is the time (week) of the i -th sample, e_i is the error of the i -th sample and has a normal distribution with mean 0 and a variance σ^2 , C_0 is the mean HOP-ratio value of the non-smokers.

pathogenic point of view, to determine the time course of recovery from the increased HOP-ratio. That is, this study was carried out with the object of bringing out a coherent association between urinary HOP and smoking and a consistence of availability of urinary HOP as marker.

The effect of cessation from smoking was assessed in 49 smokers who participated in an anti-smoking course using the urinary HOP: creatinine ration (HOP-ratio). Urine samples were collected daily at the beginning of the course for five days and during the subsequent 14 weeks, two times a week. The subjects were divided into five groups depending on the number of cigarettes smoked daily before cessation: 1-10, 11-20, 21-30, 31-40 and > 41 cigarettes. The urinary HOP-ratio immediately after cessation of smoking was proportional to the mean daily number of cigarettes smoked in the past. This result was in agreement with that of a similar survey undertaken in adult men, in the same district in the same year (Fig. 3). All subgroups showed decreasing HOP-ratios with an increasing period of abstinence. Half of the total observed decrease in the HOP-ratio after the 14 weeks was reached within 5 or 6 weeks (Fig. 4). When using the Brinkman Index to adjust for the number of smoking years, half of the maximum decrease in all subgroups was reached within four weeks. In an exponential decay model fitted to the data (Table 2), the half-life time to reach the non-smokers urinary HOP levels was nine to ten weeks for all subgroups. And it was estimated that the HOP levels of smokers who smoked less than 41 cigarettes before cessation reached the HOP levels of the control group after 61-74 weeks. The period needed for the most heavy smokers subgroup was estimated to be 120 weeks. The results suggest that the urinary HOP-ratio is useful as a biochemical marker for the short-term breakdown of lung collagen and lung elastin.

Influence of Automobile Exhaust and ETS (Environmental Tobacco Smoke) in Areas Alongside Main Road [20]

This present study is a critical assessment of the effects on health of ETS and NO_2 generated from main roads. A hydroxyproline:creatinine ratio (HOP-ratio) was used as the representative measure of the health effects. The associations between the HOP-ratio and ETS, and the HOP-ratio and low concentration NO_2 are essentially very weak; for

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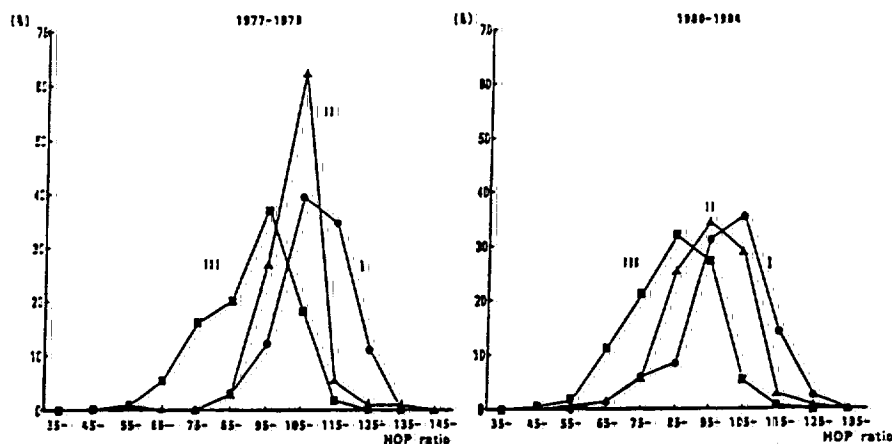


Fig. 5. Distribution of HOP-ratio by area in non-passive smokers

Remarks: Area I: within 50 m from roadside.

Area II: 51-100 m from roadside.

Area III: over 101 m from roadside

this reason, being able to control the confounding factors and their resulting bias was a major priority in the study. In order to ascertain the consistency and strength of these associations and coherence of the evidence repeated cross-sectional study was performed each year in May over an eight-year period from 1977 to 1984. 4,375 schoolchildren from F primary school area acted as subjects for the study. The school area was a typical urban residential area adjoined by main roads with a daily traffic of 3,000 vehicles or more such as C highway, but excepting these, it was a typical residential area with no other significant NO_2 generating sources as large-scale factories. The area was divided into three according to the distance from the main roads. (Area I: areas within 50 m from the roadside, Area II: within 50-100 m, Area III: over 101 m.) Subjects were divided in four groups according to levels of ETS exposure through passive smoking in the home, which were represented by the number of cigarettes smoked per day at home, by all members of the family, (NPS group: non-passive smoking, LPS group: 1-10 cig./day, MPS group: 11-20 cig./day, HPS group: 21 or more cig./day).

The association between ETS and the HOP-ratio was investigated as that of pupils living in Area III which was free of any direct influence from automobile exhaust diffusion. The area and HOP-ratio association was observed using the NPS group which was free of ETS influence.

- 1) The HOP-ratio increased as the level of ETS exposure increased and was greatest in schoolchildren who lived in the area nearest the main roads. The strength of the association was considered statistically significant based on the magnitude of the HOP-ratio, the correlation coefficient, a two-way layout and relative risk. This, with the coherence of the measurements for each area in each year, seemed to suggest without exception a strong causal relation. The influence of ETS and area distinctions on the HOP-ratio were totally independent and showed virtually no interaction.
- 2) Lowered ventilation rates in the winter heating period tended to produce high levels of NO_2 indoor air pollution and often overrode the influence of the roads (according to

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Table 3. Annual trend of HOP-ratio by ETS and by area

Area	Year	N-PS			L-PS			M-PS			H-PS			Total		
		N	Mean	S.D.	N	Mean	S.D.	N	Mean	S.D.	N	Mean	S.D.	N	Mean	S.D.
I	1977	30	114.86 ^{##}	9.44	16	119.24 ^{##}	8.85	25	124.55 ^{##}	7.59	27	133.61 ^{##}	9.50	98	123.21	11.57
	1978	33	115.68 ^{##}	8.24	13	116.84 ^{##}	11.13	22	127.07 ^{##}	7.61	22	130.72 ^{##}	13.69	90	122.31	12.08
	1979	44	110.79 ^{##}	7.71	21	117.61 ^{##}	4.67	22	121.58 ^{##}	5.32	17	122.22 ^{##}	5.64	104	116.32	8.10
	1980	16	101.73 ^{##}	13.63	8	112.85 ^{##}	17.88	16	114.67 ^{##}	12.94	13	119.32 ^{##}	21.86	53	111.63	17.87
	1981	19	108.06 ^{##}	13.14	7	112.42 ^{##}	19.95	22	114.91 ^{##}	12.69	32	122.91 ^{##}	14.71	80	116.26	15.56
	1982	20	108.56 ^{##}	11.46	6	116.26 ^{##}	5.77	17	115.18 ^{##}	9.37	16	116.41 [*]	7.42	59	113.38	10.01
	1983	35	106.34 [*]	5.38	12	107.94 [*]	5.36	21	111.06 ^{##}	5.10	20	115.45 ^{##}	6.37	88	109.76	6.61
	1984	28	96.90 [*]	11.64	12	102.82 [*]	7.48	24	103.40 [*]	12.56	16	106.37 [†]	11.76	80	101.63	12.01
	Total	225	108.55	9.73	95	113.82	10.25	169	116.68	9.57	163	122.20	12.21	652	114.84	11.840
II	1977	27	106.54 ^{##}	9.06	23	111.30	8.98	23	124.77 ^{##}	16.02	19	125.66 ^{##}	5.59	92	116.23	10.78
	1978	39	108.21 ^{##}	9.55	23	113.52 ^{##}	3.57	16	118.24 ^{##}	5.24	12	127.45 ^{##}	9.99	77	113.98	10.87
	1979	48	104.85 ^{##}	4.22	12	111.37 ^{##}	3.63	17	111.25 ^{##}	4.11	16	119.31 ^{##}	5.31	93	109.35	6.89
	1980	22	97.16 ^{##}	10.41	6	98.51	17.23	16	105.28	14.68	11	112.27 ^{##}	19.54	55	102.69	15.87
	1981	30	97.39 ^{##}	12.30	11	101.02	13.89	23	103.65	20.06	17	108.18 [*]	19.96	81	101.93	17.23
	1982	6	106.08 ^{##}	6.27	2	103.02	2.57	5	106.16	4.81	6	106.00	7.81	19	105.75	6.22
	1983	43	100.49 ^{##}	6.24	25	101.46	6.94	32	104.51 ^{##}	4.63	22	106.41 [†]	4.38	122	102.81	5.74
	1984	44	97.19 ^{##}	11.26	13	100.86	6.84	13	109.40 ^{##}	13.45	23	106.05 [†]	9.00	93	101.60	11.60
	Total	259	102.02	9.03	102	105.76	8.83	145	110.47	12.50	126	113.62	11.27	632	106.88	11.22

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III	1977	198	98.06	10.94	79	108.18**	9.33	71	110.51**	9.09	36	114.62**	7.63	384	104.00	10.03
	1978	372	91.75	14.12	93	97.93**	13.54	107	103.64**	7.77	61	109.69**	9.77	633	97.00	14.23
	1979	185	98.44	6.12	50	98.42	5.14	64	102.17**	4.74	43	111.15**	3.89	342	100.73	6.92
	1980	180	84.01	14.77	45	89.31*	13.19	52	100.93**	11.71	60	109.23**	14.36	337	91.82	17.26
	1981	311	86.19	10.09	109	94.51**	10.93	131	97.50**	15.70	107	100.43**	17.53	658	92.14	14.19
	1982	126	92.03	7.93	24	98.20**	7.36	28	108.30**	4.90	29	111.59**	7.49	207	97.69	10.84
	1983	140	94.28	9.83	59	98.87**	6.09	66	100.91**	5.70	60	103.38**	5.80	325	98.14	8.62
	1984	115	91.18	11.77	32	97.00**	8.64	30	95.37	13.57	28	98.79**	10.38	205	93.74	11.84
Total		1627	91.56	11.45	491	98.15	10.30	549	102.09	10.59	424	106.37	11.96	3091	96.51	12.64
Total	1977	255	100.94	12.02	118	110.29**	9.93	119	116.21**	12.62	82	123.43**	11.48	574	109.24	11.69
	1978	444	94.97	15.37	116	101.39**	14.52	145	108.80**	11.68	95	116.81**	14.45	800	101.00	16.50
	1979	277	101.51	7.71	83	105.15**	9.77	103	107.81**	9.22	76	115.35**	6.74	539	105.23	9.53
	1980	218	86.64	15.44	59	93.44**	16.60	84	104.38**	13.62	84	111.19**	16.86	445	95.52	18.50
	1981	360	88.28	11.87	127	96.06**	12.66	176	100.48**	17.03	156	105.89**	19.47	819	95.46	16.44
	1982	152	94.76	10.35	32	101.88**	9.81	50	110.43**	7.59	51	112.44**	8.16	285	101.47	12.23
	1983	218	97.44	9.76	96	100.68**	6.91	119	103.67**	6.51	102	106.40**	7.30	535	101.12	8.19
	1984	187	93.45	11.98	57	99.10**	8.40	67	100.97**	14.28	67	103.09**	10.91	378	97.34	12.45
Total		2111	94.65	12.41	688	101.44	11.59	863	106.36	12.55	713	111.27	13.72	4375	100.74	13.99

Remarks: *N-PS*: no smokers in the family, *L-PS*: smokers with 1-10 cig./day at home, *M-PS*: smokers with 11-20 cig./day at home, *H-PS*: smokers with more than 21 cig./day at home.

Compared non-passive smoking group with each passive smoking group. * $p < 0.05$, ** $p < 0.01$.

Compared III group (over 101 m from roadside) with I (within 50 m) and II (51-100 m) group. # $p < 0.05$, ## $p < 0.01$.

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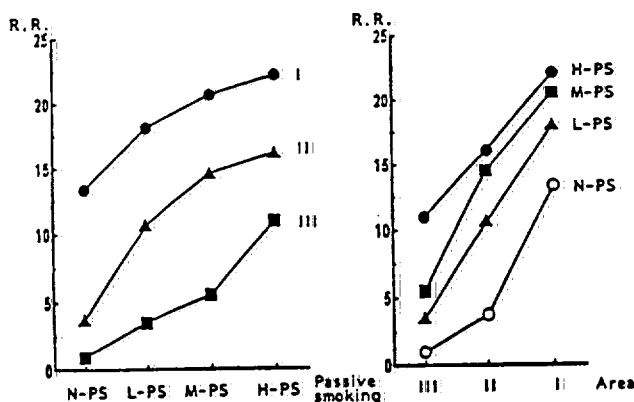


Fig. 6. R. R. of ETS and automobile exhaust (1977-1984).

Remarks: I: within 50 m from roadside, II: 51-100 m from roadside, III: over 101 m from roadside.

N-PS: no smokers in the family, L-PS: smokers with 1-10 cig./day at home, M-PS: smokers with 11-20 cig./day at home, H-PS: smokers with more than 21 cig./day at home

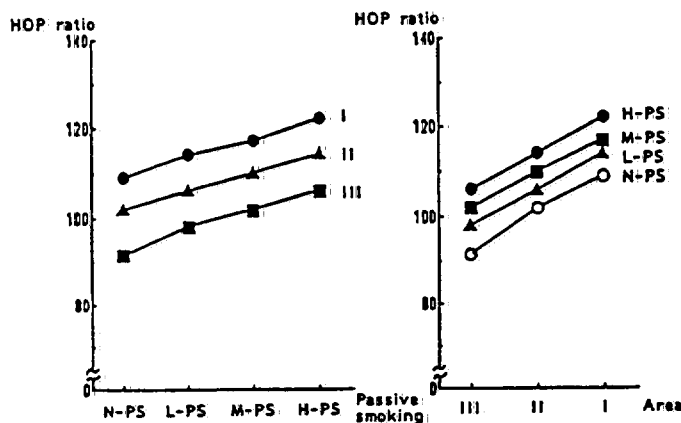


Fig. 7. HOP-ratio of ETS and automobile exhaust (1977-1984).

filterbadge measurements of NO_2 exposure levels). In addition, this not only eliminated difference in HOP-ratios according to area distinction but also produced the indoor ETS and increased ETS exposure opportunities as evidenced by the urine cotinine levels measured. It thus became clear that the survey of ETS and area distinction influence would have to be conducted during the summer months.

- 3) In order to control any bias, all pupils were subjected to a prior medical check-up and those with any confounding factors in HOP-ratios were eliminated from the study. However, the changes in patterns of indoor smoking from 1980 onwards were not sufficiently accounted for by the former interview and questionnaire surveys so we introduced an additional bias, namely misclassification of pupils. Subsequently,

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the possibility to cause underestimation of ETS influence was suggested, thereby establishing the need for a revision of the relevant questionnaires.

However, we cannot offer any conclusion on the possibility that these changes in the HOP-ratios may in the future have morbid effects to cause the development of chronic bronchitis, or emphysema of the lungs.

Impact of Smoking on the Concentration and Activity of Alpha-1-Antitrypsin in Serum in Relation to the Urinary Excretion of Hydroxyproline [21]

This study aims at assessing the coherent association between smoking and urinary HOP, in the light of the "protease and antiprotease balance theory" by Eriksson. Alpha-1-antitrypsin (α_1 -AT) is the most important inhibitor of proteases in human serum and is essential in preventing autodigestion of the lung by inhibiting elastase and collagenase. Oxidation renders this inhibitor inactive. Cigarette smoke contains many potent oxidants which can reduce the functional activity of α_1 -AT and turn the existing balance with lung proteases into an imbalance resulting in the degradation of connective tissue in the lung.

The data suggest that active smoking has significant impact on the concentration and activity of α_1 -AT in serum as well as on the urinary excretion of HOP. The data concerning passive smoking reveal less consistent results, except for the urinary excretion of HOP (Fig. 8).

The impact of active and passive smoking on the serum levels of α_1 -AT, the trypsin inhibitory capacity (TIC), the trypsin inhibitory activity (TIA) and the urinary hydroxyproline to creatinine ratio (HOP-ratio) was studied. The subjects used in the

Fig. 8. Serum α_1 -antitrypsin concentration in active and passive smokers; ** $p < 0.01$; compared with non-smokers or non-passive smokers

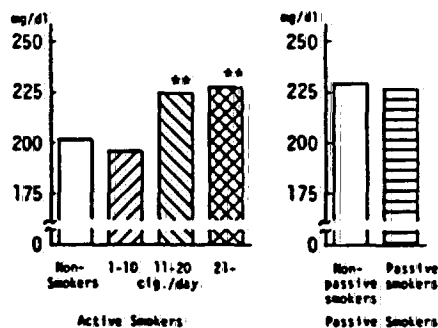
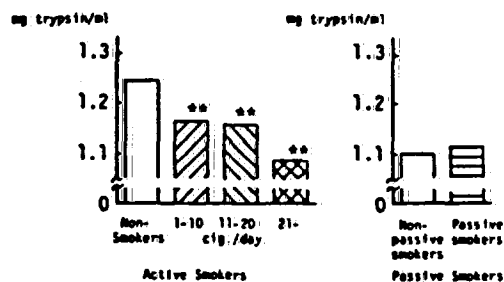


Fig. 9. Trypsin inhibitory capacity in active and passive smokers; ** $p < 0.01$; compared with non-smokers or non-passive smokers



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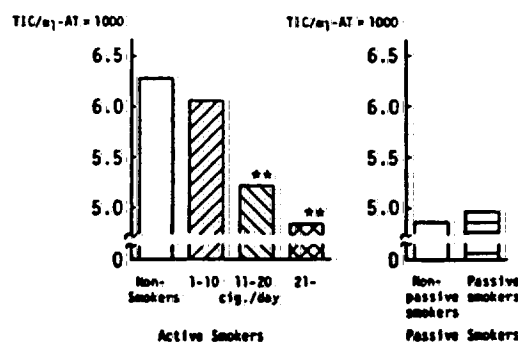


Fig. 10. Trypsin inhibitory capacity to α_1 -antitrypsin ratio in active and passive smokers; ** $p < 0.01$: compared with non-smokers or non-passive smokers

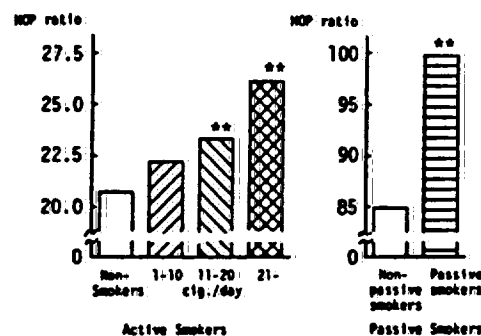


Fig. 11. Urinary hydroxyproline to creatinine ratio in active and passive smokers; ** $p < 0.01$: compared with non-smokers or non-passive smokers

study on active smoking were 167 healthy adult men and in the study on passive smoking 189 healthy primary school children. Serum levels of α_1 -AT in active smokers were significantly higher than those in non-smokers (Fig. 8). The TIC as well as the TIA in active smokers decreased with increasing number of cigarettes smoked (Figs. 9, 10). The urinary HOP-ratio increased significantly with increasing number of cigarettes smoked. On the other hand, in the case of passive smokers a significant difference was obtained only for the HOP-ratio. The associations between all markers in active smokers were significant. Less strong associations were found in the case of passive smokers (Fig. 11). These results suggest that the urinary excretion of hydroxyproline can be considered as a marker for the imbalance between proteases and antiproteases as a result of smoking.

Behavior of Urinary Hydroxyproline and Cigarette Smoking Effect in Silicosis [22]

It is common knowledge that symptoms of pneumoconiosis deteriorate rapidly with smoking. But many problems, demanding solutions from a viewpoint of epidemiology, still lie before us. It seems that the behavior of urinary HOP holds the key for them.

This study was conducted through regular pneumoconiosis examination according to the law on 1,096 employees of medium and small-sized ceramic enterprises in the Tokai district in 1981-82.

Interview examination with the BMRC questionnaire, X-ray examination and measurements of the urinary hydroxyproline to creatinine ratio (HOP-ratio) were carried

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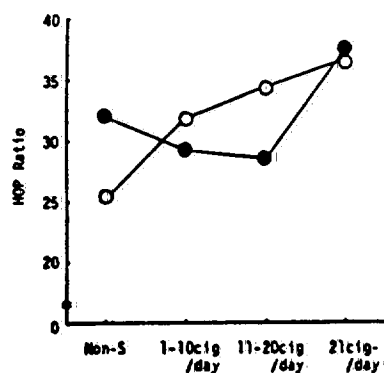


Fig. 12. Relations between urinary HOP-ratio and smoking or grade with X-ray photo. Grade with X-ray photo: O, 0; ●, 1-4.

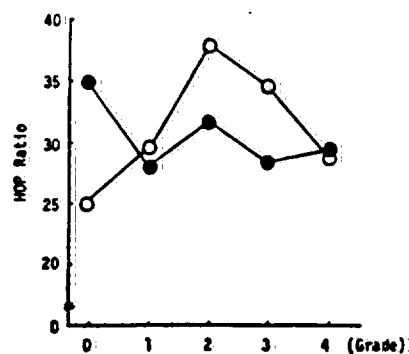


Fig. 13. Relations between urinary HOP and smoking or grade with X-ray photo. O, non-smokers; ●, smokers.

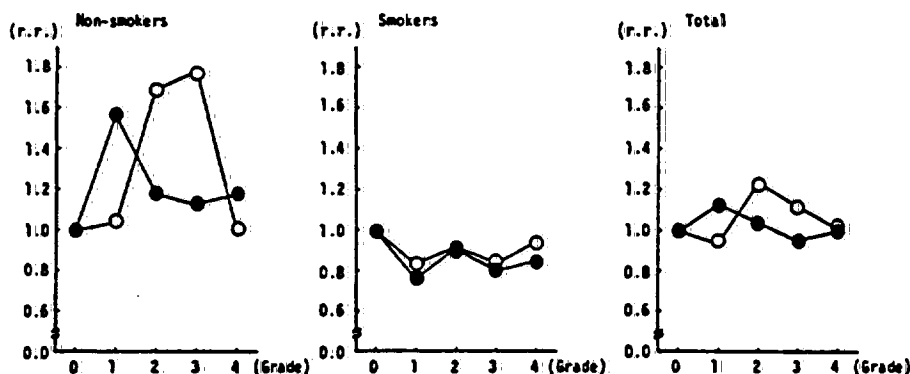


Fig. 14. Turning point of HOP-ratio by grade with X-ray photo. O, without symptoms; ●, with symptoms.

out in order to elucidate the relationship between silicosis and urinary HOP-ratio and to demonstrate the effect of smoking on pneumofibrosis. The grades of silicosis were classified into five types (0 to 4) with increasing severity of the symptoms based on the Japanese Classification of Radiographs of Pneumoconioses. Index symptoms of respiratory diseases were recorded using BMRC questionnaire [9]. In healthy subjects (type 0), the urinary HOP-ratio increased with the number of cigarettes smoked (Fig. 12).

In smokers, the collagen metabolism was rapidly repressed and fibroplastic conditions developed, although smoking itself did not seem to induce pneumofibrosis.

In the non-smoking group, the HOP-ratio was the lowest in type 0 and it increased in the order of type 1 and 2. The turning point was at type 2 and the HOP decreased type 3 and 4, by turns (Fig. 13). Further analysis of the data showed that the turning point for non-smokers without index symptoms was found at type 3, whilst the turning point for

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non-smokers with index symptoms was at type 1 (Fig. 14). Shifting of the turning point suggests that index symptoms also promote fibroplastic activities.

Contrary Opinions Against the Relationship Between Urinary HOP and Smoking, ETS and NO₂

Intervention Studies

Intervention studies of urinary HOP levels in rodents were conducted by Mullenae et al. [16], Read and Thornton [17] and Higashi et al. [18]. These studies were generally performed using an extreme short exposure term combined with an extreme small number of subjects, and they ended in failure to show a positive result.

If an exposure level of NO₂ as in actual air pollution is used, a prolonged exposure experiment with numerous subjects shall be needed from a pathological viewpoint. In addition to our study, Mizoguchi and Yoshida [23] also reported in 1986 that urinary HOP-ratios in schoolchildren living in three areas with different ambient NO₂ concentrations in Tokyo increased with the NO₂ concentration.

Opinions Presented by Adlkofer [24, 25]

- 1) Adlkofer stated in 1983 as follows: even though smoking was originally regardless of the HOP excretion, the HOP: creatinine ratio increased with smoking because the creatinine excretion was positively correlated with the number of cigarettes smoked.
- 2) He raised another objection to our studies in 1987.
 - The urine volume in smokers was larger than that in non-smokers.
 - HOP excreted into urine was reabsorbed to some extent but tubular reabsorption of HOP was interfered by the increasing urine flow.
 - In contrast with HOP, urinary creatinine was not reabsorbed at all. Therefore, he concluded that the HOP: creatinine ratios in smokers were higher, irrelevant to the higher HOP excretion.
- 3) In the same paper, he stated as follows; HOP in 24 h urine of smokers was found to be larger than that in non-smokers, but after standardizing for body surface, the urinary HOP excretion was almost completely unaffected by tobacco uptake.

Answers

- 1) If his opinion was right, it would be impossible to apply the cotinine ratio in random urine as a substitute of cotinine in 24-h urine. It is not too much to say that the usefulness of urinary cotinine: creatinine ratio was established by the report on involuntary smoking by Surgeon General U.S.A. in 1986 [28]. Urinary HOP: creatinine ratio which was developed in the 1960s by Allison et al. [26] and Whitehead [27] is prevailing now in the field of epidemiology, and it was also included in the manual on passive smoking, IARC, in 1987.
- 2) His syllogistic conclusion does not seem to be supported with high reliability and consistency. The possibility of his opinions is only very slight.
- 3) Excretion of HOP decreases with aging, and body surface increases with aging [3]. In conclusion, I think that there is no need to adjust for body surface, in this case but, it is necessary to adjust for growth or age.

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The Significance of Urinary Hydroxyproline Excretion in Smokers and Passive Smokers

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Summary

The urinary hydroxyproline/creatinine ratio was reported to be increased after smoking, passive smoking and exposure to ambient air polluted with nitrogen dioxide. In two field studies we tried to verify the results obtained in smokers. A weak positive association was found between the hydroxyproline/creatinine ratio and cigarette consumption for male smokers. In contrast, the amount of hydroxyproline excreted in the 24-h urine was not increased in male and female smokers as compared to their non-smoking counterparts. Standardizing the data for body surface led to the same results. From the findings obtained in smokers we conclude that passive smoking does not lead to an elevation of hydroxyproline excretion either.

In addition, we measured lower amounts of creatinine excreted in the 24-h urine of male, but not female smokers and higher 24-h urine volumes in smokers of both sexes as compared with non-smokers. Whereas an inverse correlation was found between the extent of smoking and the excretion of creatinine, the association between the extent of smoking and urinary volume was positive. Both findings acting together may explain the increased hydroxyproline/creatinine ratio as seen in smokers. Therefore the hydroxyproline/creatinine ratio is not a measure of the hydroxyproline excretion in smokers, nor is its determination an appropriate method of detecting lung damaging effects due to smoking and passive smoking.

Introduction

Increased urinary excretion of hydroxyproline is an established diagnostic marker for certain osteopathic destructions, some endocrinological disorders and severe burns [10]. However, only controversial results are available when hydroxyproline excretion is used to indicate degradation of lung collagen and elastin in subjects with lung diseases [9, 16]. Kasuga and co-workers introduced the hydroxyproline/creatinine ratio as a biomarker for exposure to pollutants with lung damaging properties in epidemiological field studies. They reported dose-related increases in the hydroxyproline/creatinine ratio of smokers, passive smokers and subjects exposed to ambient air polluted with automobile exhaust [9]. In an earlier investigation involving male smokers of cigarettes, pipes and cigars and passive smokers [2], we were unable to confirm the results of Kasuga and co-workers. Instead of this, we found an inverse correlation between urinary creatinine concentration and smoke uptake in cigarette, pipe and cigar smokers which could have caused the observed weak association between smoking and the hydroxyproline/creatinine ratio. In order to shed new light on this controversy we extended our study by a group of 120

subjects consisting of 60 smokers and 60 non-smokers. Hydroxyproline and creatinine in 24-h urine samples were independently measured in the laboratory of Prof. Kasuga and in our Munich laboratory.

Subjects and Methods

Subjects

All subjects were recruited as described elsewhere [2]. The data of cigarette smokers and non-smokers of study 1 in this paper are those of study 1 and 3 of an earlier publication [2]. In study 2, 60 non-smokers (30 males, mean age: 27.8 years; 30 females, 28.1 years) and 60 smokers (31 males, 31.3 years; 29 females, 30.8 years) were investigated. The subjects collected 24-h urines before they came to the laboratory between 4 and 7 p.m. On this occasion blood samples were drawn and questionnaires on life style factors were completed.

Analytical Methods

Hydroxyproline and creatinine in the urine samples of study 1 and 2 were analyzed in the laboratory of Prof. Kasuga at Tokai University, School of Medicine, by autoanalyzer techniques [8, 15]. The samples from study 2 were additionally analyzed in our laboratory using the method of Prockop and Udenfriend for hydroxyproline [12] and Jaffe's reagent (Merckotest 3385, Fa. Merck, Darmstadt, W.-Germany) for creatinine. Carboxyhemoglobin (COHb) was measured with a CO-Oximeter (Instrumentation Laboratories Ltd, Model 182) immediately after taking the blood samples. Cotinine in serum was determined by gaschromatography (study 1) [6] and radioimmunoassay (study 2) [5].

Results

The 24-h urine parameters for smokers and non-smokers of both studies are summarized in Table 1. Male smokers of study 1 showed significantly higher hydroxyproline/creatinine ratios than did male non-smokers. In study 2, the same trend is obvious for male and female subjects if Kasuga's data (TDS) are used, but could not be seen with our data (MDS). There was no difference in the amount of hydroxyproline excreted in the 24-h urine either standardized for body surface or unstandardized between male smokers and male non-smokers. In female smokers, the hydroxyproline excretion rate was similar to that in female non-smokers if our data set was used, but was significantly higher with Kasuga's data. In both studies significantly lower amounts of creatinine were measured in the 24-h urine of male smokers as compared to male non-smokers. No such difference was seen for female subjects. Our results are in line with the Japanese data set. In study 2, in both sexes the volume of the 24-h urine of smokers was significantly higher than that of non-smokers.

The relationship between the smoke uptake variables (cigarette consumption, COHb, serum cotinine) and the 24-h urine variables (hydroxyproline, creatinine, volume) are shown in Table 2. Notably, the extent of smoking is positively correlated with the 24-h urine volume and negatively correlated with the amount of creatinine excretion over 24 h. This is also true for female smokers, even if their average amount of creatinine excreted

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Table 1. Hydroxyproline (HOP) and creatinine (CREA) excretion in 24-h urine of smokers and non-smokers (mean \pm SD)
(TDS = Tokyo Data Set; MDS = Munich Data Set)

Smoking status (n)	HOP (mg/24 h)		HOP (mg/24 h/m ²)		HOP/CREA (mg/g)		CREA (mg/24 h)		Volume (ml/24 h)
Study 1 (TDS)									
Males									
Non-smokers (23)	32.3 ± 11.5		16.9 ± 6.2		17.3 ± 4.0		1,863 ± 481		1,416 ± 511
Smokers (88)	34.7 ± 16.5		17.7 ± 6.2		25.5 ± 8.5		1,373 ± 549		1,318 ± 595
p	0.41		0.62		0.0001		0.0001		0.44
Study 2									
Males									
	TDS	MDS	TDS	MDS	TDS	MDS	TDS	MDS	
Non-smokers (30)	41.5 ± 17.6	29.2 ± 10.8	22.8 ± 11.6	16.5 ± 8.2	19.5 ± 11.9	16.3 ± 5.4	2,310 ± 623	1,822 ± 490	1,399 ± 380
Smokers (31)	40.0 ± 14.6	26.2 ± 9.7	20.6 ± 8.2	13.5 ± 5.1	23.2 ± 9.9	17.1 ± 5.5	1,832 ± 508	1,543 ± 385	1,846 ± 710
p	0.73	0.26	0.40	0.09	0.19	0.55	0.01	0.02	0.01
Females									
Non-smokers (30)	29.4 ± 9.4	21.7 ± 9.3	17.5 ± 5.6	12.8 ± 5.1	22.9 ± 8.0	21.2 ± 8.3	1,326 ± 300	1,051 ± 273	1,209 ± 524
Smokers (29)	37.8 ± 17.7	20.8 ± 7.0	22.8 ± 10.9	12.8 ± 5.5	29.9 ± 16.7	19.0 ± 5.1	1,331 ± 293	1,100 ± 233	1,611 ± 846
p	0.03	0.67	0.03	0.98	0.06	0.23	0.95	0.46	0.04

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Table 2. Coefficients of correlation (Pearson) between variables of cigarette smoke uptake and 24-h urinary hydroxyproline (HOP) and creatinine (CREA) excretion and urinary volume (TDS = Tokyo Data Set; MDS = Munich Data Set)

Study 1 (TDS), Males (N = 88)

Variables	Consumption (Cig/d)	Serum cotinine (ng/ml)	COHB (%)
HOP (mg/24 h)	0.07	0.19	0.14
CREA (mg/24 h)	-0.08	0.07	-0.07
Volume (ml/24 h)	0.23*	0.19	0.27**

Study 2

Variables	Consumption (Cig/d)		Serum cotinine (ng/ml)	
	TDS	MDS	TDS	MDS
<i>Males (N = 31)</i>				
HOP (mg/24 h)	-0.11	-0.38*	0.13	-0.03
CREA (mg/24 h)	-0.44*	-0.40*	-0.24	-0.33
Volume (ml/24 h)		0.34		0.08
<i>Females (N = 29)</i>				
HOP (mg/24 h)	0.05	-0.19	-0.14	-0.29
CREA (mg/24 h)	-0.33	-0.34	-0.62***	-0.69***
Volume (ml/24 h)		0.36		0.14

* $p < 0.05$; ** $p < 0.01$; *** $p < 0.001$.

was not found to be different from that of non-smokers (Table 1). No stable trend of a correlation between the amount of hydroxyproline excreted and the smoke uptake variables could be observed.

Discussion

This investigation deals with the question of whether urinary hydroxyproline may in fact be regarded as a validated biochemical marker for assessing health hazards due to tobacco smoke exposure. The increase in urinary hydroxyproline caused by degradation of lung collagen and elastin is thought to be very low. This is due to the fact that hydroxyproline containing lung proteins constitute only some 2% of the total collagen present in the entire human organism [1]. Therefore very high turnover rates in the lung would have to be assumed if measurable increases in urinary hydroxyproline excretion were to result. It is thus not surprising that in several studies including our own no increase in hydroxyproline excretion was found after smoking [2, 7, 13] or after exposure to nitrogen dioxide [11]. Similar conclusions must be drawn from investigations of

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subjects with lung disease [10, 14]. Our present data again support the view that urinary excretion of hydroxyproline is not sensitive enough to detect a lung damaging effect of cigarette smoking, as we do not find an increase in hydroxyproline excretion in male and female smokers. As far as male smokers are concerned, our data are in line with those of Kasuga. In female smokers, Kasuga's data show a significantly higher hydroxyproline excretion. At present, we have no explanation of this discrepancy.

If there is no measurable difference in urinary hydroxyproline excretion over 24 h between smokers and non-smokers, the question arises whether the increased hydroxyproline/creatinine ratio in smokers as found in several studies might be caused by factors other than an elevation of hydroxyproline. First of all, the interlaboratory comparison shows that the hydroxyproline/creatinine ratio is subject to a high analytical variability. On the average, higher hydroxyproline levels were found in Kasuga's laboratory as compared with our laboratory (regression line is parallel to the ideal line).

The coefficient of correlation was rather low ($r=0.76$). A better correlation was observed for the creatinine measurements ($r=0.96$). However, the regression line systematically deviates from the ideal line. Due to this interlaboratory variance, the hydroxyproline/creatinine ratios calculated from the data obtained in both laboratories show a weak correlation only ($r=0.27$).

Furthermore, our data demonstrate an increase in the 24-h urinary volumes in relation to the extent of smoking. The data of study 1, in which smokers and non-smokers have similar urine volumes, may at first sight not support this finding. However, it has to be considered that in this study the smokers were investigated in winter and the non-smokers in summer which makes a comparison rather meaningless, since people use to drink more in summer than in winter. We must assume that the urinary flow rate influences excretion of hydroxyproline and creatinine in a different way. Whereas a nearly complete tubular reabsorption is reported for free hydroxyproline which accounts for 80-95% of total hydroxyproline in the plasma [1], no tubular reabsorption of creatinine takes place at all. According to the principle of forced diuresis, an increased urinary flow rate as found in smokers may cause a reduction in tubular reabsorption of hydroxyproline, thus leading to an increased urinary excretion. This remains to be established by experimental evidence.

Finally, the evaluation of the data sets clearly shows that the urinary creatinine excretion is inversely correlated with the extent of smoking. So far we cannot offer any convincing explanation of this finding. However, our observation is supported by at least two papers. One of them reports lower creatinine concentration in serum of smokers as compared with non-smokers [3]. The other describes lower serum creatinine levels in male but not in female smokers as compared to their non-smoking counterparts [4]. Therefore the increased hydroxyproline/creatinine ratio if found in smokers might, for the most part, be caused by a diminished urinary creatinine excretion.

In conclusion, increases in the hydroxyproline/creatinine ratios in smokers might be falsely interpreted as an elevated excretion of hydroxyproline. This may in fact be caused by smoking-related decreases in creatinine excretion and/or increases in urine volume. Moreover, hydroxyproline excretion is unlikely to be an appropriate biomarker for detecting a lung damaging effect of smoking or passive smoking. Low increases in urinary hydroxyproline after smoking, which cannot be excluded, may remain undetected due to the wide interindividual and methodological variabilities, and in view of the high release of hydroxyproline from organs other than the lung.

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DNA Adducts, Protein Adducts, and Sister Chromatid Exchange in Cigarette Smokers and Nonsmokers^{1,2}

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ABSTRACT—In order to validate markers of internal dose and biologically effective dose of carcinogens, a battery of measurements was made on blood samples from 22 smokers and 24 nonsmokers. The markers included immunoreactivity in an enzyme-linked immunosorbent assay (ELISA) quantified in white blood cells with the use of a polyclonal anti-benzo[a]pyrene diol epoxide-I-DNA antibody, 4-aminobiphenyl hemoglobin (4-ABP-Hb) adducts measured by negative chemical ionization mass spectrometry, sister chromatid exchange (SCE) in cultured lymphocytes, and cotinine in plasma measured by radioimmunoassay. Several blood samples were drawn from each subject. In blood samples 1 and 3 having detectable levels of DNA adducts, mean femtomole-per-microgram levels were consistently higher among smokers compared to nonsmokers. The borderline significance of this difference may be attributable to the small numbers of subjects. Consistently higher adduct levels were seen in females compared to males. In sample 1, adduct levels were significantly correlated with measurement of active smoking in smokers and with passive smoking in nonsmokers. By contrast to the ELISA data, which may reflect cumulative exposure from multiple background sources, the 4-ABP-Hb assay was able to distinguish clearly between smokers and nonsmokers. SCEs were significantly elevated in the smokers compared to nonsmokers. Also observed were significant correlations between 4-ABP-Hb and both cotinine and SCEs, as well as a significant correlation between the 4-ABP-Hb and DNA adduct levels (sample 3) that was highly significant. The correlation between DNA and 4-ABP-Hb adducts was significant in smokers but not nonsmokers (sample 3). These results support the need for batteries of markers to detect and to quantify the carcinogenic dose to humans resulting from both specific and "background" environmental exposures.—*JNCI* 1987; 79:449-456.

Methods to quantify biologically effective dose (the amount of activated carcinogen interacting with critical cellular targets) can greatly enhance the epidemiology of human carcinogenesis and can also improve risk extrapolation between species (1, 2). Carcinogen-DNA adducts measured by immunoassays (2-6), postlabeling (7), and physical methods (6) are viewed as a particularly useful and relevant marker of biologically effective dose in human populations. This approach is supported by evidence that covalent binding to DNA is a critical early event in the process of tumorigenesis (8) and that the carcinogenic potency of a number of PAHs correlates with their ability to form specific DNA adducts (9, 10).

Incomplete combustion of organic materials, including fossil fuels, is the major source of PAHs, such as BP, BA, and chrysene. For example, BP is a ubiquitous pollutant encountered in the workplace, urban air, drinking water, and food supply (11, 12). It is a constituent of mainstream (20-40 ng/cigarette) and sidestream cigarette

smoke (68-136 ng/cigarette) (13). Long understood to be a carcinogen in active smokers, cigarette smoke is now increasingly recognized as contributing to lung cancer in nonsmokers (13-16).

Antibodies elicited against BPDE-I-DNA were used to establish a sensitive ELISA (17). They were later shown to cross-react with DNA modified by diol epoxides of other PAHs that form adducts with stereochemistry similar to BPDE-I-DNA, such as BA (with a 20-fold lower affinity than BP) and chrysene (with a twofold to threefold lower affinity) (Santella R, Harris C: Personal communication). These two PAHs are found in the same sources as BP, in fairly similar concentrations (e.g., BA, 26 ng/cigarette; chrysene, 66-96 ng/cigarette) (12). Thus positive reaction with the antibody may indicate the presence of multiple PAH-DNA adducts. Measurement of these other DNA adducts is biologically important, because, like BP, both BA and chrysene are

ABBREVIATIONS USED: 4-ABP = 4-aminobiphenyl; 4-ABP-Hb = 4-ABP adducts in hemoglobin; BA = benzantracene; BP = benzo[a]pyrene; BPDE-I-DNA = BP diol epoxide-I-DNA; ELISA = enzyme-linked immunosorbent assay; GC-MS = gas chromatography-mass spectrometry; NCIMS = negative chemical ionization mass spectrometry; PAH = polycyclic aromatic hydrocarbon; ppd = packs/day; SCE = sister chromatid exchange.

¹ Received December 5, 1986; accepted April 21, 1987.

² Supported by Public Health Service grants 5R01CA-35809 and 5R01CA-39174 from the Division of Extramural Activities, National Cancer Institute; and by grant CU50267401 from the American Lung Association.

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⁷ Deceased March 6, 1987.

⁸ We thank Dr. I. B. Weinstein, Director, Comprehensive Cancer Center, Columbia University, and Director, Division of Environmental Sciences, Columbia School of Public Health, for his invaluable advice, support, and encouragement throughout this project. We also appreciate the assistance of Dr. R. Lewy, Director, Employee Health Center, Presbyterian Hospital, and his staff. Our thanks to L. Levine for her work on questionnaire development; S. V. M. ELISA analysis, and Dr. H. Melani for statistical assistance. All are associated with the Columbia University School of Public Health. Finally, we are grateful to J. Roby for assistance with manuscript preparation.

mutagenic and carcinogenic (18). In addition, this polyclonal antibody reacts with BPDE-I-RNA, but with tenfold lower reactivity than for BPDE-I-DNA (Poirier MC, Santella RM, Haas R: Unpublished observations).

The anti-BPDE-I-DNA antibody was used to analyze human samples in a pilot study of lung cancer cases and controls (4) and subsequently to detect DNA adducts in human placental tissue (19). The present study is an effort to relate adduct levels and two additional complementary markers of biologically effective dose—carcinogen-protein adducts and SCE—to cigarette smoking and other environmental exposures.

4-ABP is another potent carcinogen and mutagen (20) found in mainstream (2–4.6 ng/cigarette) and sidestream (140 ng/cigarette) cigarette smoke (13, 21). Background sources include air pollution, azo dyes, and possibly charred meat but are lower than for PAHs (22). Thus 4-ABP-Hb is a useful marker of exposure to cigarette smoke. Protein-carcinogen binding is considered a useful surrogate for DNA-carcinogen adducts (23, 24); in particular, hemoglobin can act as a trap for carcinogens, providing a cumulative dosimeter over its 4-month lifetime (25). Sensitive GC-MS techniques are available for quantitating 4-ABP levels on hemoglobin. These techniques have been validated in experimental animal studies showing that rats dosed with 4-ABP formed a stable, accumulating covalent 4-ABP-Hb adduct (25). Here, in collaboration with Dr. M. Bryant, Dr. S. Tannenbaum, and Massachusetts Institute of Technology co-workers, the NCIMS method was applied for the first time to quantitate 4-ABP-Hb adducts in humans. The data presented here are the initial data in a larger series (22, 26).

SCEs are viewed as a useful marker of biologically effective dose of mutagens-carcinogens (27, 28). Prior studies have shown significant differences in SCEs between smokers and nonsmokers (27). As a generic marker, SCE is complementary to the other two chemical-specific techniques.

Finally, in collaboration with Dr. N. Haley of the Naylor Dana Institute for Disease Prevention, cotinine levels [a metabolite of nicotine with a plasma half-life of 10–13 hr (29, 30)] were measured in the plasma of a subset of subjects. Cotinine is a highly specific biochemical marker of internal dose of cigarette smoke, both active and passive (13, 29, 30).

The goal of the present pilot study was to compare the three complementary markers of biologically effective dose and to relate each to an objective measure of cigarette smoking (cotinine as a tobacco-specific dosimeter). Detailed personal exposure and health histories were obtained by questionnaire and compared to the marker data. These histories were designed to elicit information about host and environmental factors known or believed to contribute to PAH or 4-ABP adducts and SCEs.

MATERIALS AND METHODS

Subjects, sample collection, and questionnaire data.—Twenty-two healthy smokers and 24 nonsmokers who

were either employees or students at the Columbia Presbyterian Medical Center, New York, NY, volunteered for the study. This group was selected because there was no anticipated significant occupational exposure to the pollutants of interest. Nonsmokers were defined as individuals who had never smoked cigarettes, but could have smoked cigars, pipes, or marijuana. Three of the nonsmokers admitted to smoking marijuana 2 years or more prior to study, whereas another 2 reported current usage. Because the mean assay values of nonsmokers as a group did not change to any significant degree when these individuals were factored out of the analysis, they were retained in the nonsmoking group. The same was true for 2 former cigar and pipe smokers. Marijuana smoking among cigarette smokers also did not substantially change the group mean.

All subjects were interviewed by trained personnel by means of a standardized questionnaire. Indices of active smoking were: lifetime tar (sum of mg tar/cigarette for each brand of cigarette smoked during lifetime [based on Federal Trade Commission tar values for cigarettes 1956–84 (31)] \times number of cigarettes smoked of that brand/day \times 365 \times number of years smoked that brand), current smoking levels (ppd), pack-years (ppd \times yr smoked), and tar levels of current brand smoked (31). A passive smoking score reflecting cumulative residential exposure during the previous 2 years was calculated (No. of persons smoking \times their ppd \times No. of hours of subject exposure/day \times No. of months living with smokers in past 2 yr). The average total daily hours individuals were exposed to passive smoke at home and work during the preceding 2 years were also included in the analysis. A charcoal exposure score incorporating both average intake during the previous 2 years and intake during the previous 2 weeks reflected consumption of broiled or smoked meat and fish. In addition to these quantitative scores, participants were qualitatively rated on area of residence (urban vs. rural) as a measure of outdoor air pollution, recent exposure to wood smoke or kerosene heaters, occupational and environmental exposures to PAHs or to other substances capable of influencing SCE formation (e.g., petroleum products, tar, and ethylene oxide), and other forms of smoking (e.g., cigars, pipes, and marijuana). Information on alcohol and caffeine consumption, medications, x-rays, and health status-history was collected to identify potential confounders in the SCE analysis. A more complete analysis of the SCE data, taking into account these possible confounders, will be presented separately (Munshi AA, Fischman HK, Perera F: Manuscript in preparation).

Nonsmokers gave two blood samples and smokers gave three blood samples of 35 ml each. For smokers, the second sample was drawn 17–18 hours after the first sample, and the third was drawn 48 hours after sample 1. For the nonsmokers, the second sample was drawn 48 hours after the first. Samples were coded and centrifuged; buffy coat cells, hemoglobin, and plasma were stored frozen at -70°C . In addition, some subjects gave an additional 10 ml for SCE analysis.

Competitive ELISA for BPDE-I-DNA adducts.—The general method has been described (4) and will only be briefly summarized here. Microplates (96 wells) were coated with 5 ng BPDE-I-DNA (0.5% modified). The rabbit polyclonal antibody to BPDE-I-DNA was used at 1:100,000 dilution, and the goat anti-rabbit IgG alkaline phosphatase conjugate was used at a dilution of 1:500. The standard curve was established with the use of known amounts of BPDE-I-DNA in 50 μ g carrier calf thymus DNA, with a mean 50% inhibition of 23.2 fmol/50 μ g. For the biologic samples, combined DNA (native) and RNA were prepared as previously described (32) with extensive proteinase K and phenol treatment to eliminate protein. The ratio of absorbance at 260 and 280 nm was used to monitor purity. Combined RNA and DNA was used so that larger amounts of material would be available for repeated assays.

Samples were assayed at 50 μ g/well and run in duplicate wells (average coefficient of variation = 7%). Sample percent inhibitions were calculated, and the corresponding femtomole-per-microgram equivalents of BPDE-I-DNA were determined from the standard curve. While the assay is measuring multiple PAH-DNA adducts, since the standard curve used in vitro modified BPDE-I-DNA, data are expressed as fmol BPDE-I-DNA, which would give the equivalent percent inhibition (or "BPDE-I-DNA antigenicity"). DNA adducts were measured in white blood cells from 22 smokers and 24 nonsmokers (sample 1), in 20 smokers (sample 2), and in 21 smokers and 21 nonsmokers (sample 3). We considered samples with less than 25% average percent inhibition to have nondetectable levels of BPDE-I-DNA antigenicity.

NCIMS for 4-ABP-Hb adducts.—The method is described in detail elsewhere (25, 26). The GC-MS procedure involves hydrolysis of the cysteine sulfinamide adduct of 4-ABP followed by derivatization with pentafluoropropionic anhydride to yield the pentafluoropropionamide, which was then analyzed by GC-MS with the use of negative chemical ionization. 4-ABP-Hb adducts were measured in red blood cells from 19 smokers and 18 nonsmokers (sample 1) and in 9 smokers and 6 nonsmokers (sample 3).

SCEs.—After separation of leukocytes from the plasma, duplicate 72-hour cultures per subject were done as previously described (33). Bromodeoxyuridine was added at 24 hours. Coded slides were prepared and stained according to the method of Goto et al. (34). Fifty metaphases were analyzed per culture, and the average number of SCEs per metaphase was calculated. SCEs were measured in cultured lymphocytes from 11 smokers and 10 nonsmokers.

Cotinine.—Cotinine in plasma was quantitated by a modified radioimmunoassay as described elsewhere (30). This method is able to determine cotinine levels as low as 1 nmol/ml plasma. All samples with cotinine levels below this limit were listed as nondetectable. Cotinine values were measured in plasma from 10 smokers and 10 nonsmokers.

Statistical methods.—The principal statistical approaches were to compare the 2 groups (smokers and

nonsmokers) with respect to results in each assay and then to evaluate correlations between a number of host and exogenous variables and respective assay values. To assure comparability of samples, the two samples from nonsmokers were compared to the first and last samples (#1 and #3) taken from smokers. For both groups, these were the samples drawn 48 hours apart. Half of the smokers were asked to abstain from smoking for this 48-hour period to investigate short-term decay of adducts. Since compliance with this request was highly variable and we saw no consistent effect of reducing or quitting smoking during this time period in the subset of 11 subjects who cooperated, the data from this group were not analyzed separately. The 2-group comparisons were based on standard two-tailed *t*-tests. *P*-values less than .01 were considered highly significant; those between .01 and .05, significant; and those between .05 and .1, borderline.

Pearson's method was used to analyze correlations between assay results and various exogenous and host variables. These included measures of active and passive smoking (cumulative lifetime tar, pack-years, average packs/day smoked during lifetime, level of tar in current cigarette, residential passive smoking score, and average total hours exposed to passive smoke/day) as well as age and dietary exposure to charcoal. Finally, the various assays were compared to determine how well they correlated with each other. Here significance was evaluated using a two-tailed *t*-test for the transformed correlation: $r/\sqrt{1-r^2/n-2}$, where *r* is the correlation coefficient and *n* is the sample size. This method was selected because it takes account of small sample size.

RESULTS

The mean age of the smokers was 35.7 (± 9.3); for the nonsmokers it was 32.9 (± 9.8). The smokers included 12 females and 10 males; nonsmokers included 12 females and 12 males. Smokers were all currently consuming at least 1 pack per day (mean = $1.4 \pm .4$) and had lifetime smoking histories ranging from 7.5 to 62 pack-yr (mean = 24.5 ± 15.1). Table 1 shows the results for each assay for smokers and nonsmokers, respectively.

BPDE-I-DNA antigenicity.—Of the 46 subjects studied, 5/22 (22.7%) smokers and 7/24 (29%) nonsmokers had detectable levels of adducts in sample 1. For sample 3, the proportion of detectables was 4/21 (19%) for smokers and 4/21 (19%) for nonsmokers. All statistical analyses were performed on this smaller subset of individuals whose samples showed detectable adduct levels. The mean results for this subset are summarized in table 2.

Mean adduct levels were consistently higher in smokers than nonsmokers (samples 1 and 3). This difference showed borderline significance in sample 3. Females were also consistently higher than males in both samples. Significant correlations were seen among smokers in sample 3 with both average ppd smoked (.995) and lifetime tar intake (.964); a borderline correlation was seen with pack-years (.925). In sample 1, the equivalent

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TABLE 1.—Age, sex, and assay values for volunteers^a

Patient			ELISA, ^b fmol/ μ g DNA			4-ABP, ^c pg/g Hb	SCE, ^d average No./ metaphase	Cotinine, ^e ng/ml
No.	Age, yr	Sex	Sample 1	Sample 2	Sample 3			
Smokers								
3	45	F	ND	ND	.067	105	NA	491
4	40		ND	.154	ND	95	NA	545
5	52		.144	.186	ND	75	NA	446
8	45		.210	.114	.198	217	NA	491
11	21		ND	ND	ND	160	NA	274
12	33		ND	NA	NA	NA	NA	NA
14	34		ND	ND	ND	256	NA	145
15	31		ND	ND	ND	179	NA	549
16	31		ND	ND	ND	164	NA	400
17	27		ND	ND	ND	232	NA	440
21	38		ND	ND	.123	98	12.8	581
23	32		ND	ND	ND	142	10.5	NA
25	30		ND	ND	ND	125	10.7	NA
27	48		.111	NA	ND	213	11.3	NA
33	27		ND	ND	.067	NA	7.2	NA
36	39		ND	ND	ND	163	7.7	NA
39	37		ND	ND	ND	156	12.1	NA
40	22		ND	ND	ND	143	10.1	NA
41	34		ND	ND	ND	135	11.7	NA
44	48		ND	ND	ND	NA	NA	NA
49	23	.100	.133	ND	146	13.8	NA	
50	50	.100	ND	ND	141	11.1	NA	
Patient			ELISA, ^b fmol/ μ g DNA		4-ABP, ^c pg/g Hb	SCE, ^d average No./ metaphase	Cotinine, ^e ng/ml	
No.	Age, yr	Sex	Sample 1	Sample 3 ^d				
Nonsmokers								
0	43	F	ND	ND	29	NA	ND	
1	35		.155	.103	35	NA	ND	
2	32		.125	ND	7	NA	ND	
6	26		ND	ND	NA	NA	NA	
7	43		.170	ND	17	NA	ND	
9	41		ND	ND	42	NA	3	
10	49		ND	ND	34	NA	ND	
13	27		ND	ND	30	NA	ND	
18	23		ND	ND	20	NA	ND	
19	46		ND	ND	47	10.6	ND	
20	27		ND	ND	45	7.9	ND	
24	29		ND	ND	48	9.1	NA	
26	53		ND	NA	11	9.0	NA	
28	24		ND	.045	24	8.8	NA	
30	23		ND	ND	49	7.7	NA	
31	23		.097	.062	36	6.0	NA	
32	48		.088	.050	NA	7.0	NA	
34	27		ND	ND	22	5.9	NA	
35	23		ND	ND	33	9.1	NA	
42	28		.085	NA	NA	NA	NA	
43	44		.085	NA	NA	NA	NA	
45	25		ND	ND	NA	NA	NA	
46	23		ND	ND	NA	NA	NA	
48	38		ND	ND	42	12.5 ^e	NA	

^a NA = not assayed, ND = not detectable. Hb = hemoglobin.^b Expressed as fmol equivalents of BPDE-I-DNA adducts/ μ g DNA.^c Results for sample 1 only.^d A total of two samples was drawn from nonsmokers. Because the second was equivalent in time to the third sample drawn on nonsmokers, the second nonsmoker sample was labeled sample 3 to facilitate analysis.^e Subject excluded from SCE analysis because of known exposure to ethylene oxide.

TABLE 2.—Mean assay results: smokers and nonsmokers.

Assay	Smokers	Nonsmokers
ELISA, fmol, μ g DNA ^a		
Sample 1	.133 \pm .047	.115 \pm .036
Range	.100-.210	.085-.170
No. detectables assayed	5/22	7/24
Sample 2	.147 \pm .030	Not done
Range	.114-.186	
No. detectables assayed	4/20	
Sample 3	.128 \pm .054 ^b	.065 \pm .026
Range	.067-.198	.045-.103
No. detectables assayed	4/21	4/21
4-ABP-Hb, pg/g hemoglobin		
Sample 1	154.5 \pm 49.3 ^c	32.2 \pm 12.3
Range	75-256	7-51
No. assayed	19	18
Sample 3	139.0 \pm 23.7 ^c	36.2 \pm 12.3
Range	84-163	14-51
No. assayed	9	6
SCE, average No./metaphase		
Sample 1 ^d	10.8 \pm 2.0 ^d	8.1 \pm 1.5
Range	7.2-13.8	5.8-10.6
No. assayed	11	10
Cotinine ng/ml		
Sample 1 ^e	419.2 \pm 149.9 ^c	0.3 \pm .1
Range	145-581	0-3
No. assayed	10	10

^afmol equivalents of BPDE-1/ μ g DNA.^bSmoker/nonsmoker differences: .05 < *P* < .1.^c*P* = .0001.^d*P* = .002.

correlation coefficients were .290, .659, and .474. There was also a significant correlation among nonsmokers with the two passive smoking variables in sample 3: .963 for passive residential score and .963 for total hours exposed.

4-ABP-Hb.—As shown in table 2, there was a highly significant difference between the mean 4-ABP-Hb levels of the smokers and nonsmokers (samples 1 and 3). For all subjects, 4-ABP-Hb was significantly correlated with indices of active smoking: pack-years (.63), cumulative lifetime tar (.57), average packs/day (.80), and tar level of current cigarette (.77). In the 2 groups combined, a borderline significant correlation (.37) was seen between one of the measures of passive smoking (total hours exposed/day) and hemoglobin adduct levels. However, these correlations are primarily accounted for by the highly significant difference in adduct levels between the smokers and nonsmokers.

SCEs.—Mean SCE levels for smokers were significantly higher than those for nonsmokers (table 2). In both groups combined, a highly significant correlation was seen between SCEs and average packs smoked/day (.61). Significant correlations were seen between levels of SCEs and lifetime tar intake (.45) and between pack-years (.48) and tar level of current cigarette (.44). As with the 4-ABP-Hb adducts, these findings were not replicated in the smoker group when analyzed separately and are probably due to the differences in means between non-smokers and smokers.

Cotinine.—There was a highly significant difference between the mean cotinine concentrations of smokers

and nonsmokers (table 2). For all subjects, the amount of smoking as measured by lifetime tar intake (.78), pack-years (.82), average packs/day (.89), and tar level of current cigarette (.84) showed statistically significant positive correlations with cotinine levels. However, these correlations appear to be primarily due to the differences between the smokers and nonsmokers.

Relationships between the various assays.—Table 3 shows the correlations between the assays run in smokers and nonsmokers combined. As can be seen, the two ELISA data sets (samples 1 and 3) were highly inter-correlated. Highly significant correlations were found between 4-ABP-Hb and cotinine and between 4-ABP-Hb and SCE level. A significant relationship was found between DNA adducts (sample 3) and SCEs.

Due to small numbers, it was not possible to directly compare DNA and protein adducts in sample 3. Since there was little difference between 4-ABP-Hb values in the repeat samples (average coefficient of variation = 14%), we compared sample 1 4-ABP-Hb values to DNA adduct levels in both samples 1 and 3. An interesting finding was the significant correlation of DNA adduct levels in the third sample with 4-ABP-Hb adducts. This is consistent with the observation that this sample showed strong correlations with indices of active smoking in smokers and with passive smoking in nonsmokers. When the relationship between DNA and protein adducts was examined separately for smokers and nonsmokers, correlation coefficients for smokers were always positive and exceeded those of nonsmokers.

DISCUSSION

We have assayed human blood cells for three complementary markers of internal and biologically effective doses of mutagens-carcinogens in cigarette smoke. They included highly sensitive chemical-specific methods (immunoassays and NCIMS) and a generic assay (SCE). Of interest to us were the relationship between the individual markers and both objective and subjective estimates of exposure on the one hand and comparisons between the various dosimeters on the other.

TABLE 3.—Interassay correlations^a

Specification	ELISA		4-ABP	Cotinine	SCE
	Sample 1	Sample 3			
ELISA—	—				
Sample 1					
ELISA—	.978 ^b	—			
Sample 3	(4)				
4-ABP	-.026	.944 ^b	—		
	(8)	(5)			
Cotinine	.504	.470	.583 ^b	—	
	(5)	(4)	(20)		
SCE	.567	.818 ^c	.658 ^b	—	—
	(5)	(5)	(19)	(3)	

^aNumbers in parentheses are the numbers of subjects involved in each comparison.^b*P* < .01.^c*P* < .05.

The levels of DNA adducts in samples with detectable amounts (0.025–0.21 fmol/μg) were in the same range as in our prior study of lung cancer patients (0.14–0.18) (4) and considerably lower than those reported in various worker populations (5, 6). This is consistent with the different PAH exposure levels for these populations but may also reflect the different methods of sample and DNA preparation.

Here we are using white blood cell DNA as a surrogate for the target tissue (lung). Similar adduct levels have been observed in lymphocytes and lung DNA of experimental animals treated with BP (35), as well as in human lung and white blood cell DNA in a limited number of lung cancer patients [(4) and unpublished observations]. The extent of DNA modification by cisplatin has also been comparable in white blood cells and various other human tissues (36).

Limitations of our study design are that, with the exception of the 17 individuals having cotinine measurements, the estimates of exposure rely on self-reported history of smoking and other relevant factors obtained by questionnaire. Second, comparisons between the markers in terms of sensitivity, specificity, persistence, etc. are hindered by the small number of individuals with a full battery of measurements. Third, the markers are not strictly comparable because they involve different cell types, tissues, or chemical constituents with variable lifetimes and repair capacity (29, 37). Finally, our study is limited to measurement of adducts formed by PAHs in cigarette smoke; however, recent studies have suggested the importance of persistent tobacco-specific nitrosamine-DNA adducts with respect to the carcinogenic potential of cigarette smoke (38, 39). Thus it would be desirable also to measure O₆-methylguanine adducts in smokers and nonsmokers.

A consistent increase was observed in mean DNA-adduct levels in smokers compared to nonsmokers. In fact, in the smokers (sample 3) several indices of active smoking were significantly correlated with adduct levels. The significant correlation between adduct levels and passive smoking also observed in the nonsmokers (sample 3) adds support to recent reports of increased cancer risk among individuals exposed to passive smoke (16). As in previous studies using immunoassays with an antibody to BPDE-DNA (4–6), detectable levels of adducts were observed in some but not all of the smokers having comparable smoking exposure (1½ ppd). Although inter-assay variability may be a contributing factor, this observed interindividual variability suggests considerable differences in PAH metabolism and/or adduct repair and is consistent with the wide range in PAH binding observed in human tissues *in vitro* (40). In sample 3, where smokers' DNA adducts were clearly related to smoking, the proportion of samples with detectable levels of DNA adducts (19%) is also consistent with the observation that only 10–20% of heavy smokers develop lung cancer (41).

The surprisingly high frequency of adducts observed among nonsmokers may be attributable to the ubiquity of PAH exposure (including passive smoking, diet,

occupation, and indoor and outdoor air pollution). The large majority of subjects lived in New York City or nearby New Jersey, an urban environment with high background levels of PAHs.

4-ABP-Hb levels were significantly higher in the smokers than nonsmokers. However, significant correlations between the various indices of active passive smoking were not seen when the smokers were analyzed separately. This may be due to the relatively small number of smokers.

The highly significant correlation between DNA and 4-ABP-Hb adducts in sample 3 is of interest because in this sample, unlike sample 1, DNA adducts were also strongly related to reported consumption of cigarettes. In addition, when smokers and nonsmokers were analyzed separately, the correlation between DNA and protein adducts was highly significant for smokers (sample 3) but not for nonsmokers. In sample 1, this correlation was positive though nonsignificant in smokers and negative in nonsmokers. Since 4-ABP is a more direct marker of smoking than PAH-DNA adducts, as confirmed by the highly significant correlation between 4-ABP and cotinine, we conclude that BPDE-I-DNA antigenicity in the smokers, especially in sample 3, was primarily attributable to smoking.

SCE levels in smokers and nonsmokers were significantly different; however, there was no relationship between the amount of active smoking and SCEs in smokers. SCEs were significantly correlated with 4-ABP-Hb and with DNA adducts in sample 3. The lack of a dose response between smoking and SCEs is not consistent with a prior report of an increase in mean SCE frequency with the number of cigarettes smoked per day in 42 individuals (42). Our relatively small number of smokers (*n*=11) may have prevented our seeing this effect.

This pilot study aimed to validate several complementary markers of biologically effective dose of PAHs in cigarette smokers and nonsmokers. Smokers can be considered a "model" population whose external exposure can be estimated with reasonable accuracy. The study established that the methods used are adequately sensitive for human studies and that it is critical in molecular epidemiologic studies to account for "background" exposures. The study also illustrated the advantage of using a battery of markers—some specific to a chemical or source—others capable of reflecting total genetic dose from environmental exposures. The battery should also reflect a range of exposure periods and patterns, e.g., current as well as past or cumulative exposure.

Finally, we observed significant differences in adduct levels and SCEs in individuals with comparable exposure. These were not unexpected given the known interindividual variation in absorption and activation-deactivation of carcinogenic chemicals and in the efficiency of DNA repair systems (40, 43–47). In fact, these observed differences in markers may reflect variance in risk of developing cancer. However, while evidence of genetic damage indicates a potentially increased risk of cancer, quantitative predictions on the individual level must

await data from longitudinal studies definitively linking the specific markers to cancer risk. We hope that this pilot study will encourage further detailed molecular epidemiologic investigations of markers in model populations.

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³²P-postlabelling analysis of DNA adducts in monocytes of smokers and passive smokers

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Received October 23, 1989 / Accepted March 9, 1990

Summary. In a controlled study, ten male volunteers were subjected to different smoking and passive smoking conditions. After 60 h of strictly controlled nonsmoking, five smokers were exposed to mainstream smoke only, while five nonsmokers were exposed to the gas phase of environmental tobacco smoke (ETS). In a second experiment smokers were mainstream and ETS exposed, while nonsmokers were exposed to complete ETS. Blood was drawn before and after smoking and DNA adducts were analysed from blood monocytes by the ³²P-postlabelling assay, using the nuclease P1 enhancement method. We detected DNA adducts in monocytes of all probands. These adducts unrelated to smoking showed interindividual differences but only minor intraindividual changes in four samples of the same donor. After smoking interindividually variable additional adducts were visible in active smokers only. These smoking-related adducts had disappeared after 40 h of nonsmoking and reappeared again in three out of five smokers after the second smoking period. We conclude that smoking causes an interindividually variable pattern of DNA adducts in monocytes (smokers). These adducts disappear in less than 2 d. owing to the fast turnover of monocytes in the intravascular system. The effects described could not be observed in heavily exposed passive smokers.

Key words: Postlabelling assay – Smoking – Passive smoking – Monocytes

Introduction

Although a series of suspected carcinogenic substances have been found in cigarette smoke, there is still no defi-

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nite answer as to which specific compounds are responsible for the increased risk of smokers in acquiring lung cancer [6]. In addition, there is a growing need to evaluate a possible carcinogenic risk in passive smokers, suggested by several epidemiological studies, although the extent of the effect remains to be investigated [16]. Since the covalent binding of xenobiotics or their metabolites to DNA is an important indicator for genotoxic stress [15], we studied the formation of DNA adducts in monocytes of smokers and passive smokers. The ³²P-postlabelling assay, being one of the most sensitive methods, seems to be especially suited for analysing the formation of DNA adducts resulting from the exposure to complex mixtures of unknown composition [17]. After pretreatment with nuclease P1 the nucleotides carrying a covalently bound adduct are selectively labelled with ³²P-phosphate. This allows the detection of PAH or bulky adducts with a sensitivity of a few molecules per genome [5]. Though several studies using the ³²P-postlabelling assay have shown a higher level of adducts in several tissues of smokers as compared to controls [12, 14], this has not yet been possible with human white blood cells [10, 11]. Here we used isolated monocytes, because these cells are metabolically active without pretreatment [1] and are therefore expected to reflect the formation of DNA adducts *in vivo*.

Materials and methods

Design of the study. Ten male healthy volunteers, five smokers (age 19–28) and five nonsmokers (age 23–29) refrained from smoking 60 h before the first blood sample was drawn. During the 6 d of the study the probands received a standardized diet, equal in amount and quality. Two different exposure conditions were applied: The third day smokers were exposed to mainstream smoke only and the fifth day to mainstream smoke and ETS; nonsmokers were exposed to the gas phase of ETS for 8 h on the third day and to complete ETS for 8 h on the fifth day. In the first experiment the five smokers had to smoke a total of 24 cigarettes (1 cig./20 min) and exhaled the smoke through an one-way-valve into the exposure

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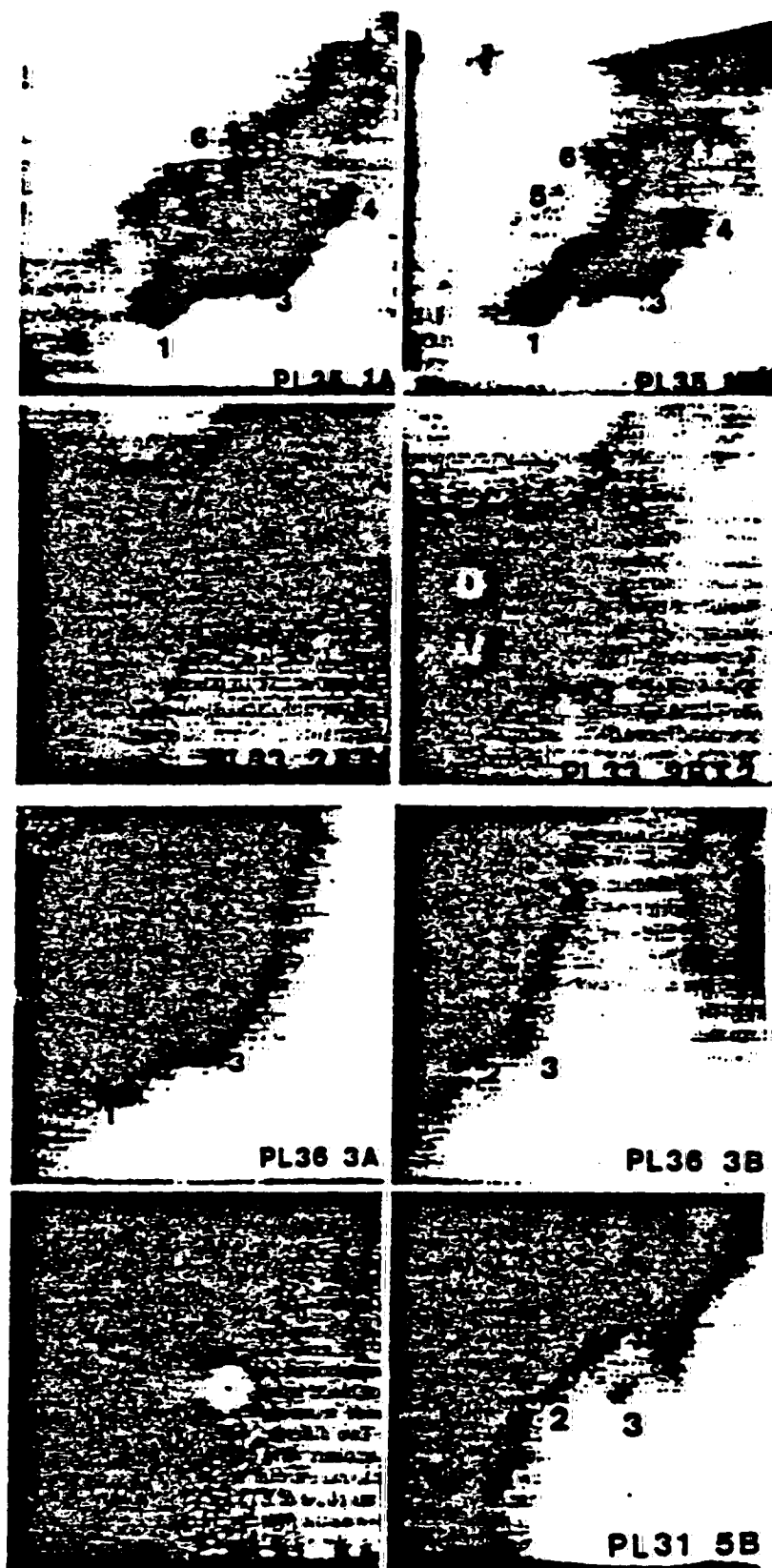


Fig. 1. Autoradiographs of PEI-cellulose t.l.c. maps of ^{32}P -labelled digest of DNA from peripheral blood monocytes of smokers (probands 1, 2, 3, 5). Sample A taken before and sample B taken after the first smoking period. R1, R2, R4, R5 are additional spots detected after smoking. Screen intensified autoradiography at -80°C for 72 h

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Fig. 2. Autoradiographs of PEI-
cellulose i.l.c. maps of Δp -la beiled
digest of DNA from peripheral
blood monocytes of a smoker
(proband 4). Samples taken before
and after the first (A, B) and the
second (C, D) smoking period.
Smoking related adducts are only
visible after smoking (R1, R2).

resolved by 4-dimensional chromatography on PEI-cellulose t.l.c. sheets (Macherey-Nagel, Düren, FRG). The plates were first developed with 1M sodium phosphate, pH 6.0, to wash polar adduct-free nucleosides on to a paper wick. The paper wick was removed and the plates were developed again in 3.5M lithium formate, 8.5M urea, pH 3.5, and after turning the plates 90° in 0.8M lithium chloride, 0.5M Tris-HCl and 8.5M urea, pH 8.0. Subsequently the plates were developed in 1.7M sodium phosphate on to a paper wick again to lower the background activity on the plates. The adducts were detected by screen intensified autoradiography for 72 h at -80°C (Kodak 5, Kodak, Rochester, NY, USA).

Results

Tobacco smoke exposure of the subjects is clearly reflected in significant increases in carboxyhemoglobin (COHb) after the 8-h exposure sessions. On Day 3 of the study, COHb increased by 7.1% in the active smokers and by 2.2% in the passive smokers. The corresponding increases for Day 5 were 7.7 and 2.6%.

The cells of all probands showed various DNA adducts which exhibited interindividual differences in amount and chromatographic behavior but were unrelated to smoking (sample A). After the first 8-h period of smoking additional DNA adducts appeared in samples of all smokers (sample B). These smoking related adducts varied individually (Fig. 1). In monocytes from sample C, (i.e. after a 40-h nonsmoking period), these additional adducts had disappeared, while the nonsmoking related adducts remained. After the second 8-h smoking experiment the additional adducts were detected again in samples of three smokers (Fig. 2). Nonsmokers exposed to ETS (passive smokers) did not show any additional adducts. There was no difference between samples taken after exposure to the gasphase only (sample B) and taken after exposure to total ETS (sample D).

Discussion

Smoking caused DNA adducts in peripheral blood monocytes of active smokers. Passive smoking did not lead to similar adducts. Since the samples were analysed using the nuclease P1 enhancement method it can not be concluded that passive smoking did not cause any DNA adducts at all. Nuclease P1 also dephosphorylates nucleotides carrying smaller and rather polar adducts, which for instance may arise due to tobacco specific nitrosamines. Therefore by the method used here, predominantly PAH derived or other bulky adducts are likely to be detected. Smoking-related DNA adducts in monocytes had already disappeared after a 40-h nonsmoking period. This is probably due to the relative short lifespan of blood monocytes ($t_{1/2} = 8$ h) [7] in the intravascular system and might explain why other authors [10, 11] were not able to detect differences between smokers and nonsmokers on peripheral blood leucocytes. Studies on lung tissue [12] and mouse skin [13] have shown a rather long half life of bulky DNA adducts, so that is unlikely to attribute the observed rapid disappearance of smoking-related adducts to a fast reacting repair mechanism in human monocytes.

All samples exhibited adducts not related to smoking (adducts 4-7). Similar adducts were not observed in fibroblast DNA, probably because these cells are cultivated under standardized conditions, and because pre-existing adducts were diluted by multiple cell proliferation. Monocytes seem to mirror the actual amount of genotoxic stress and showed DNA adduct level in almost all samples analysed in our laboratory. The adducts 1, 2 and 3 (Fig. 1), however, which were found in almost all samples analysed in this study could also be found in fibroblast DNA, while calf thymus DNA never showed these spots. Therefore we assume that culture conditions may have caused these adducts. In addition, since their provenance remains finally unexplained, we do not make any attempt here to speculate about variable intensities of these spots. Other adducts in monocytes unrelated to smoking, however, must be either rather fast developing adducts since monocytes display a fast turnover in blood, or may represent pre-existing adduct levels in monocyte stemcells. Both, smoking-related adducts and unrelated adducts exhibited intraindividual variations. This could be explained by genetically determined differences in the amounts of various enzymes being responsible for metabolic activation or detoxification of exogenous and endogenous substances in the cell [3, 4, 8, 9].

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Cytogenetic effects of tobacco smoke exposure among involuntary smokers

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(Received 23 December 1987)

(Revision received 14 April 1988)

(Accepted 25 April 1988)

Keywords: Involuntary smokers; Chromosome damage; Transplacental exposure

Summary

Tobacco smoke is highly genotoxic and produces chromosomal damage in several experimental systems. Active smokers have been shown to have an increased prevalence of somatic chromosome damage in their peripheral blood lymphocytes: this is seen in most cases as an increased sister-chromatid exchange (SCE) frequency and often also as increased structural chromosome aberrations (CAs). Among passive smokers, in association with exposure to environmental tobacco smoke, no such induction of chromosomal damage has been documented. In the present paper we report negative results on induction of chromosomal damage in 2 separate groups of intensive involuntary exposure to tobacco smoke, non-smoking restaurant personnel and newborn children of smoking mothers. While significant exposure in these groups is clearly seen in biochemical intake markers, e.g. cotinine and thiocyanate values in plasma, the conventional cytogenetic parameters, structural chromosome aberrations and sister-chromatid exchanges, are unable to detect the low exposures of involuntary smokers.

Most involuntary smoking occurs through exposure to environmental tobacco smoke (ETS), i.e., passive smoking. The chemical and biological characteristics of ETS have been discussed in this volume by Löffroth, Claxton and collaborators. Transplacental exposure of the unborn child is also, in fact, involuntary even if the chemical composition of the exposing agent, tobacco smoke, is different from ETS; it mainly consists of the transplacental components of main-stream smoke inhaled by the actively smoking mother.

The well-documented genotoxic character of tobacco smoke condensates and all forms of tobacco smoke, main-stream, side-stream and environmental tobacco smoke in experimental conditions (see e.g., IARC, 1986; Claxton et al., this volume), also makes the exposure measures that are specific to the genotoxic character applicable in the natural exposure situation. In this report, we especially discuss the use of cytogenetic exposure measures, i.e., structural chromosome aberrations (CAs) and sister-chromatid exchanges (SCEs) in peripheral blood lymphocytes, to detect involuntary exposure to tobacco smoke in heavily exposed passive smokers and in newborn babies of smoking mothers.

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0165-1218/89/\$03.50 © 1989 Elsevier Science Publishers B.V. (Biomedical Division)

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Subjects and methods

The occupationally exposed group comprised 16 non-smoking waiters and 9 smoking waiters working in a night restaurant where there was no restriction in smoking. As controls, 7 non-smoking persons without obvious recent or past exposure to environmental tobacco smoke were included. A detailed description of the work-site restaurant (restaurant B) of the subjects was given in an earlier publication (Husgafvel-Pursiainen et al., 1986). The non-cytogenetic exposure parameters of the subjects (together with personnel from 2 other restaurants) have been described (e.g., cotinine, thiocyanate, carboxyhaemoglobin) in Husgafvel-Pursiainen et al. (1987) and the detailed results of the sister-chromatid exchange analysis by Husgafvel-Pursiainen (1987).

The present paper reports the analysis of chromosome aberrations in these same subjects and discusses the individual concordance of the 2 cytogenetic parameters, CAs and SCEs.

For the CA analysis the whole-blood microculture method was used with a 50-h culture time (see Mäki-Paakkanen et al., 1980 for details). The scoring was performed on coded slides by an experienced analyst counting 100 metaphases per subject.

The SCE frequencies of the transplacentally exposed group were studied from cord-blood samples taken at delivery. To avoid methodological variation between culture periods, a large batch of culture medium, including bromodeoxyuridine (20 μ M; Calbiochem), phytohemagglutinin (1%; Wellcome), penicillin (100 units/ml) and streptomycin (100 μ g/ml) in RPMI 1640 (Gibco) supplemented with 15% fetal calf serum (Gibco) was prepared for the whole study and stored at -20°C . The sister-chromatid exchange analyses were performed on duplicate cultures harvested and stained according to standard procedures (see Husgafvel-Pursiainen, 1987), scoring on codes, 50 second metaphases per individual.

The mothers were interviewed for their smoking habits and possible passive exposure a few days after the delivery. The group consisted of 17 actively smoking mothers and their newborn babies and 25 non-smoking mothers and newborns; only 7 of the non-smoking mothers de-

scribed some exposure to environmental tobacco smoke. The biochemical intake markers measured from both the maternal and the cord-blood plasma samples were cotinine and thiocyanate; the analytical results and their discussion are reported separately (Sorsa et al., in preparation). In the present paper we discuss the findings of maternal-child correlations of SCEs in association with the smoking status of the mother.

Results and discussion

Chromosomal damage in passively exposed restaurant personnel

Non-smoking personnel working in indoor restaurants without restrictions of smoking of the public or personnel probably represents one of the groups most heavily exposed to ETS at work. The environmental monitoring data, including analysis of polyaromatic compounds, total particulate matter and genotoxic activity of particulate samples, from typical night restaurant show high levels of contamination of the indoor air (Husgafvel-Pursiainen et al., 1986). The biochemical intake markers of tobacco smoke, i.e., cotinine and thiocyanate, also show significantly increased values in the passively exposed restaurant personnel as compared with non-exposed persons (Husgafvel-Pursiainen et al., 1987). Still, these intake markers are only a few percent of the values found in the actively smoking group of waiters (Fig. 1).

Both chromosome aberrations and SCEs were analysed in a total of 32 subjects (Table 1, Fig. 2). No significant differences were seen between the

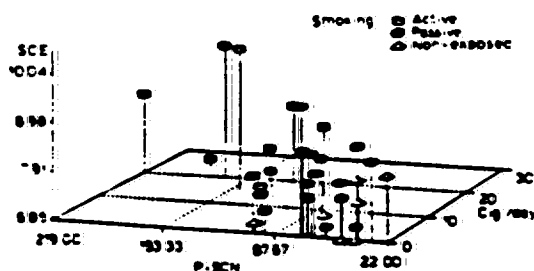


Fig. 1 Dose dependence of tobacco smoke exposure and SCE frequency among restaurant personnel, as evaluated on the basis of individual values in mean SCE/cell, thiocyanates (μ mole/l) in plasma (P-SCN) and number of cigarettes smoked daily.

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TABLE 1

MEAN LYMPHOCYTE SCEs AND MEAN PERCENTAGE OF LYMPHOCYTES WITH CHROMOSOME ABERRATIONS IN DIFFERENT SMOKING CATEGORIES

Smoking status	Number of subjects	Mean SCEs /cell (\pm SD)	% aberrant cells (incl gaps \pm SD)
Active smokers	9	8.21 \pm 1.19	5.0 \pm 1.7
Non-smokers			
Passively exposed ex-smokers	9	8.14 \pm 0.76	4.6 \pm 3.1
Passively exposed never-smokers	7	8.08 \pm 0.62	2.7 \pm 1.8
Non-exposed	7	7.47 \pm 0.59	3.7 \pm 1.6

groups or subgroups in the 2 parameters, neither was there any correlation at the individual level. In the total material, SCEs of the smoking waiters were significantly increased in comparison to non-smoking persons in the study (Husgafvel-Pursiainen, 1987). However, a clear trend in the prevalence of chromosome aberrations, especially in chromosome-type aberrations, is seen in the group of ex-smokers (mean non-smoking time after cessation of smoking was 9.6 ± 6.8 years; range 1–23 years) and smokers (Table 2). The difference is significant ($P < 0.05$) when all never-smokers ($n = 17$) are compared with current and ex-smokers ($n = 18$). However, in the larger population (Husgafvel-Pursiainen et al., 1987), neither cotinine nor SCE values of passively exposed ex-smokers (U-cot mean 52 ± 27 ng/ml, SCE mean 7.2 ± 1.3 , $n = 12$) differed significantly from the values of never-smokers (U-cot mean 60 ± 44 ng/ml, SCE mean 8.6 ± 1.1 , $n = 15$).

The significance of chromosome-type aberrations and rearrangements has been stressed earlier, since they are independent of age and sex and are

positively correlated with the duration of smoking (Vijayalaxmi and Evans, 1982; Obe et al., 1984; Littlefield and Joiner, 1986). The result also indicates the persistence of lesions leading to chromosome-type aberrations in persons who stopped smoking years ago. In comparison, the SCEs in peripheral lymphocytes of smokers decrease to normal in a few months after stopping smoking (Sario et al., 1987).

Fetal exposure to tobacco smoke

The hazards of maternal smoking to the normal development of the fetus have long been known (see e.g., National Research Council, 1986). Risks of spontaneous abortion, preterm births, prenatal deaths and low full-term birth weights have been associated with maternal smoking during pregnancy (Surgeon General, 1986). A recent case-control study suggested an association between maternal smoking and increased risk of childhood malignancies (Stjernfeldt et al., 1986). Lowering of birthweight has also been associated with heavy passive exposure of the mother to ETS (Marin and Bracken, 1986; Rubin et al., 1987), and fetal exposure caused by maternal passive smoking has

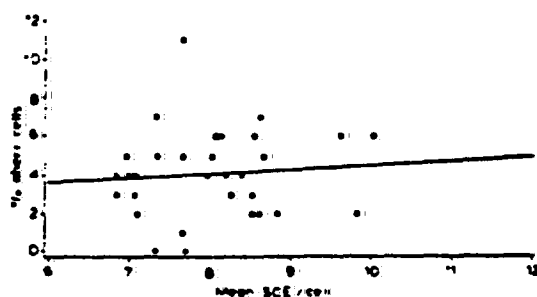


Fig. 2 Relationship of structural chromosome aberrations and SCEs ($r = 0.089$, non-significant) among the group of restaurant personnel (see Fig. 1).

TABLE 2

NUMBER OF CHROMOSOME-TYPE ABERRATIONS IN SMOKERS, EX-SMOKERS AND NEVER-SMOKERS*

	Smokers	Ex-smokers	Never-smokers
Dicentric	1/900	3/900	1/1400
Other rearrangements	3/900	2/900	1/1400
Breaks	6/900	9/900	7/1400
Total	10/900	14/900	9/1400

* Number of aberrant cells/cells analyzed

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been documented by the detection of cotinine in amniotic fluid samples (Andersen et al., 1982).

In the present study, we report the sister-chromatid exchange results in cord-blood samples taken during delivery of actively and passively exposed smokers and non-exposed non-smokers.

Biochemical intake markers measured from maternal peripheral blood just before delivery and from cord blood just after birth of the child showed transplacental concentrations of these tobacco smoke constituents to be at nearly the same level as in the mother. The correlation coefficient of maternal-cord-blood plasma cotinine was $r = 0.81$ ($P < 0.001$; 11 pairs) and for plasma thiocyanate $r = 0.92$ ($P < 0.001$; 17 pairs).

As expected on the basis of earlier studies, smoking mothers had significantly ($P < 0.01$) increased SCE values (range 7.5–10.8; mean 9.0 ± 0.9 ; $n = 17$) as compared with non-smoking mothers (range 6.7–8.9; mean 8.1 ± 0.9 ; $n = 25$). A dose-effect correlation was seen both for plasma thiocyanate value and for the number of cigarettes smoked daily (Fig. 3A).

Only 7 of the non-smoking mothers said in the personal interview that they had been exposed to ETS because of spouse's smoking. However, no significant increase of SCEs was detected in this group of passive smokers; the mean was 8.4 ± 1.0 as compared with 7.9 ± 0.9 of the non-exposed non-smoking mothers ($n = 18$).

On the basis of the biochemical intake markers, however, the small group of passively exposed persons were exposed at their homes only minimally. All of the mothers had been out of occupational ETS exposure about one month before delivery during their maternity leave.

The SCE rate in the cord-blood samples was significantly lower (mean $= 6.0 \pm 0.5$; $n = 43$) than in maternal samples (mean $= 8.4 \pm 0.9$; $n = 42$). We reported earlier also (Husgafvel-Pursiainen et al., 1980) that young children (mean age 1.5 years) have significantly lower SCE rates than adults. In earlier studies of smoking mothers and their newborn children no effect of smoking on SCEs was observed in maternal blood, while these studies also reported lower mean SCE rates in cord-blood samples (Ardito et al., 1980; Seshadri et al., 1982). A possible source of discrepancy in the maternal SCE values of smokers vs. non-smokers is the

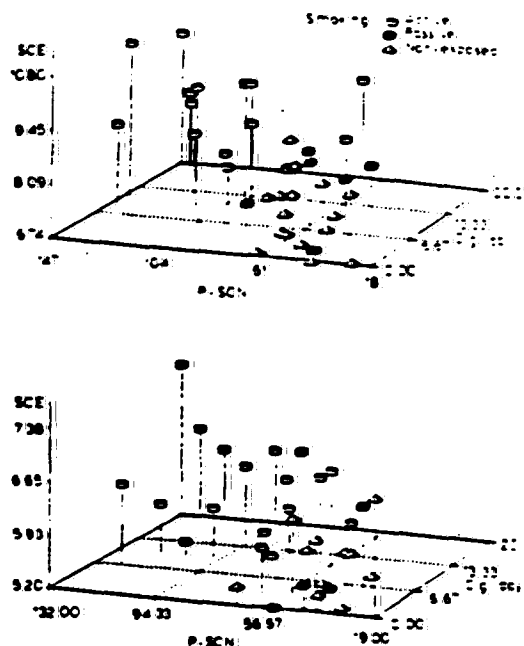


Fig. 3. Association of individual mean SCE to thiocyanate concentration ($\mu\text{mole/l}$) in plasma (P-SCN) and maternal smoking (number of cigarettes/day) in groups of smoking and non-smoking mothers (A) and newborn children (B). In A, if P-SCN and SCE were determined from peripheral blood and in B from cord blood.

bromodeoxyuridine concentration used during culturing, as shown by Lundgren et al. (1987).

In transplacentally exposed children no significant effect of maternal smoking on SCEs was observed (mean 6.1 ± 0.5 in babies of smoking mothers vs. 5.9 ± 0.5 in babies of non-smoking mothers). In the individual values, however, a clear trend of heavy maternal smoking can be seen; children with the highest plasma thiocyanate values tend to have higher SCE rates than children of mild smokers and non-smokers (Fig. 3B), although this difference is not statistically significant. Neither was there any correlation between maternal SCE value and cord-blood value, even though cotinine and thiocyanate concentrations in plasma showed highly significant correlations between maternal and cord-blood samples.

Several hypotheses can be presented to explain the low SCE rate in cord-blood lymphocytes: either the lymphocyte population responding to the

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mitogen stimulus is different in its SCE response in children and adults, or transplacental exposure to exogenous SCE-inducing agents differs from the maternal situation with regard to effective concentrations and long enough exposure time during the fetal period.

Recently accumulated evidence associates involuntary exposure to environmental tobacco smoke with health effects in non-smokers; this has been most clearly shown for respiratory organ infections among children exposed to parental smoking and for a small increase of lung cancer risk among non-smoker spouses exposed to ETS caused by the other spouse's smoking (Surgeon General, 1986; National Research Council, 1986). The biochemical intake markers usually show passive smokers' exposure to be only a few percent of the values found among active smokers (see e.g., Jarvis et al., 1984, 1985; Sorsa et al., 1985). In newborn children exposed transplacentally, the biochemical intake markers show values at nearly the same level as in their mothers, still, the fetal exposure time is only a few months.

The cytogenetic damage, well documented among active smokers (see e.g., IARC, 1986), cannot be shown to be associated with involuntary exposure to tobacco smoke in either of the two exposure situations studied, occupational or transplacental. The obvious insensitivity of the cytogenetic parameters may partly be due to their unspecificity in relation to any single compound. Also, tobacco smoke exposure may be confounded by other potentially genotoxic agents in the environment.

Acknowledgements

Grants from the National Board of Health (No. 3.1.3./86) and the Research Council for Environment Studies, Academy of Finland (No. 24/064) are gratefully acknowledged.

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MTRL 0962

Sister-chromatid exchange and cell proliferation in cultured lymphocytes of passively and actively smoking restaurant personnel

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(Accepted 21 October 1986)

Keywords: Lymphocytes, cultured; Smokers; Restaurant personnel; Sister-chromatid exchange frequencies.

Summary

Sister-chromatid exchange frequencies were measured in peripheral lymphocytes of 12 cigarette smokers, 20 passive smokers, and 14 non-smokers with no regular exposure to tobacco smoke. All active and passive smokers worked as waiters and waitresses in restaurants.

The passive smokers showed neither an increased mean SCE value nor an increased number of high SCE frequency cells (HFCs) when compared to non-exposed non-smokers. The incidence of SCEs and HFCs was observed to be elevated ($P < 0.01$; $P < 0.05$, resp.) among the active smokers.

The proliferation rate of lymphocytes in whole blood cultures from the different exposure groups was also studied. The proportion of cells in first mitosis was lower and the mean replication index (RI) higher among the smokers than among non-smoker controls. However, no significant correlation was observed between the individual mean SCE and the replication index.

Recently, IARC (1986) evaluated the literature and concluded that tobacco smoke is carcinogenic, and its activity in various short-term tests could be substantiated. Cigarette smokers show an increased frequency of sister-chromatid exchanges in peripheral blood lymphocytes as compared with non-smokers. The increase in SCE has been reported to be dose-dependent with the amount of smoking (Lambert et al., 1978; Murthy, 1979; Hopkin and Evans, 1980; Carrano, 1982; Husum

et al., 1982; Livingston and Fineman, 1983; Husgafvel-Pursiainen et al., 1984; Wulf et al., 1984).

The exposure to tobacco smoke is not limited to those who smoke, but also non-smokers may be exposed to tobacco smoke generated in the environment by smokers. Environmental tobacco smoke mainly consists of sidestream smoke, which is emitted from the burning tip of a cigarette, whereas mainstream smoke is inhaled, and partly exhaled, by the smoker. Although the exposure of non-smokers to chemically altered forms of tobacco smoke differs to some extent from that of smokers, many carcinogenic and mutagenic com-

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pounds have been identified in higher concentrations in sidestream smoke than in mainstream smoke (e.g. U.S. DHHS, 1982). Furthermore, severe effects on health, similar to those found in active smokers, have also been associated with passive smoking (see IARC, 1986, for review).

The illustrated dose-dependence of SCE increases in active smokers implies that the biological effects of a constant low-level exposure to the combustion products of tobacco in passive smoking could be detected by similar methods. The observed increase in the lymphocyte SCE of heavy or moderate cigarette smokers has, however, been small - approximately 10-30% of the non-smoker values.

Passive smoking also constitutes a health problem in places of work. In addition to known irritative characteristics of environmental tobacco smoke (Weber, 1980; U.S. DHEW, 1979), the studies linking passive smoking with lung cancer and other types of cancer have extended the problem in the work environment (U.S. DHHS, 1982; Collishaw, 1984; IARC, 1986).

This study investigates the incidence of SCEs in passive smokers as compared to that of cigarette smokers and non-exposed non-smokers. The passively exposed group consists of waiters and waitresses, most of whom have been working in smoky restaurants for several years.

In addition, proliferation of lymphocytes in whole blood cultures from the different exposure groups was studied.

Subjects and methods

The SCE analysis was performed in one group of smokers and two non-smoker groups. The smokers comprised 12 waiters and waitresses (5 men and 7 women; mean age 34.7 years, S.D. 11.3; cigarette consumption range 7-40 cig./day, mean 22 cig./day). The passively exposed non-smoker group consisted of 20 waiters and waitresses (6 men and 14 women; mean age 38.2 yrs, S.D. 9.5) who worked 40 ± 5 h per week in 3 restaurants which were heavily contaminated with tobacco smoke. 14 non-exposed office

workers (2 men and 12 women; mean age 42.1 yrs, S.D. 8.6) with no reported passive exposure either at work or at home (4 individuals reported short occasional passive exposure during working hours) were included as controls. At the time of blood sampling, health and occupational histories were obtained from personal interviews; the questionnaire data included information about sex, age, smoking status, estimation of exposure to environmental tobacco smoke, medication, radiation, recent viral infections, vaccination, diet and other life-style factors.

Blood samples were collected from the controls in the afternoon and from the waiters and waitresses in the evening, after 3-5 h of work. Whole blood cultures were set up in RPMI 1640 medium (Gibco) supplemented with 15% fetal calf serum (Gibco), 0.03% L-glutamine, 100 units/ml penicillin, 100 μ g/ml streptomycin, 1% phytohaemagglutinin (PHA; HA 15, Wellcome) and 5-bromo-2-deoxyuridine (BrdUrd; Calbiochem) at a final concentration of 15 μ M. Two replicate cultures were prepared from each sample and incubated at 37°C in the dark. At 64 h, colcemid (Gibco) was added to the cultures for 2 h. The air-dried slides were stained using a modified fluorescence plus Giemsa technique (Husgafvel-Pursiainen et al., 1980). 30-50 differentially stained metaphases each with 45-46 chromosomes were scored for SCEs per sample on coded slides by one person. The proportions of cells in first, second and third or subsequent mitoses were obtained by scoring 100 metaphase cells. To obtain the mitotic index, the number of mitoses were scored in 1000 lymphocytes per subject. Statistical evaluation was carried out using *t*-test, χ^2 -test and linear correlation.

Results

Fig. 1 shows the individual mean frequencies of SCE per cell and the range of SCE means in each group. The group mean among the active smokers was 9.06 SCE per cell (S.D. 1.1) which was significantly higher ($P < 0.01$, 2-tailed *t*-test) than the mean SCE in the non-exposed non-smoker

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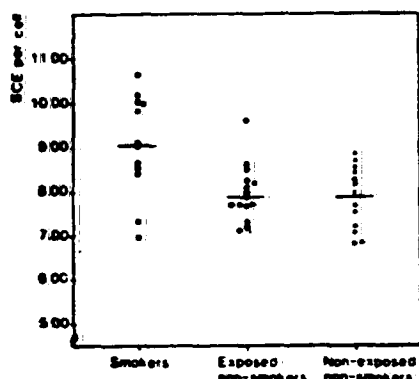


Fig. 1. The frequencies of SCE in lymphocytes of individuals with different degrees of smoke exposure. ■, cigarette smokers; ●, non-smokers exposed to environmental tobacco smoke at work; ○, non-smokers exposed both at work and at home; ▲, non-exposed non-smokers. Bars indicate the means for each group.

group (7.87 SCE per cell, S.D. 0.68). The mean SCE level in exposed non-smokers (7.92 SCE per cell, S.D. 0.65) did not differ from that observed in the non-exposed group. The incidence of SCEs in the smoker group showed a dose-dependence: the mean frequency in smokers who reported smoking more than 20 cigarettes per day was 9.4 (S.D. 0.8), and 8.6 (S.D. 1.5) in those who reported lower daily consumption (10–19 cig.).

To evaluate further the induction of SCEs related to tobacco-smoke exposure, a high frequency cell analysis was performed for each group on the pooled SCE scores (Carrano and Moore, 1982; Moore and Carrano, 1984). Based on the

TABLE 1

HIGH FREQUENCY CELLS (HFCs, CELLS WITH ≥ 15 SCEs) IN POOLED CELL POPULATIONS FOR 3 EXPOSURE GROUPS

Exposure group	Number of HFCs	Number of cells with < 15 SCEs
Active smokers	28*	332
Passive smokers	15	385
Non-exposed non-smokers	16	404

* $p < 0.05$, compared with the non-exposed controls, χ^2 -test.

pooled SCE data, the active smokers showed 28 HFCs (i.e. cells with 15 or more SCEs) as compared to 16 HFCs among the passive smokers and 15 HFCs among the non-exposed controls (Table 1). The difference between the active smokers and the controls was significant (χ^2 -test), but the number of HFCs in the passively exposed group did not deviate significantly from that of the non-exposed group.

Since most of the subjects in the study were women, the effects of active and passive tobacco smoke exposure on SCE frequency were examined when the data from the males were excluded. A very similar result was obtained; the mean SCE was 9.1 SCEs per cell for actively smoking women, 8.0 SCEs per cell for passively exposed women and 7.9 for non-smoking control women.

The proliferation rates of cells in the cultures was studied both as proportions of cells in first, second or third (or subsequent) mitosis and as a replication index derived from these data. Non-smokers had significantly more lymphocytes in first mitosis than the smokers, i.e. lymphocytes of smokers showed a faster rate of proliferation in the

TABLE 2

MEAN LYMPHOCYTE CONCENTRATIONS, MITOTIC INDICES, DISTRIBUTION OF 1st (M_1), 2nd (M_2) AND 3rd OR FURTHER (M_3 ...) METAPHASES, AND REPLICATION INDICES IN WHOLE BLOOD CULTURES

	Active smokers	Passive smokers	Control non-smokers
Lymphocytes/ml culture ($\times 10^5$)	1.79	1.60	1.13
Mitotic index	3.8	3.8	3.5
Distribution of metaphases (%)			
M_1	14.9	16.8	23.1
M_2	33.5	34.4	32.4
M_3 ...	51.8	48.7	44.4
Replication index ^a	2.4	2.3	2.2

$$1 \times M_1 + 2 \times M_2 + 3 \times M_3 \dots$$

^aRI =

100

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culture conditions. Again, the values of the passive smokers were between smokers and non-smokers. For the smokers, the mean lymphocyte count in peripheral blood was $3.57 \times 10^9/l$, and for the passive smokers and the non-smoking controls $3.19 \times 10^9/l$ and $2.57 \times 10^9/l$, respectively. Consequently, the mean number of lymphocytes per whole blood culture for each group was different (Table 2). When the data from the 3 exposure groups were pooled, no significant correlation was detected between the individual SCE frequency and the white blood cell count or the lymphocyte concentration in the cultures. Furthermore, neither the mitotic index (MI) nor the replication index (RI) correlated significantly with mean SCE frequency (SCE vs. MI, $r = 0.004$; SCE vs. RI, $r = 0.13$).

Discussion

In this study, no significant increase was observed in the lymphocyte SCE level in the group of non-smokers with a long-term passive exposure to tobacco smoke. The mean SCE frequency of cigarette smokers was significantly increased compared with both the mean SCE of passive smokers and that of the non-exposed non-smokers. Analysis of the data using the high frequency cell method similarly revealed only an increase in the smoker group, as shown previously (Carrano and Moore, 1982; Husgafvel-Pursiainen et al., 1984); the number of HFCs in the passive smokers did not differ from the pooled baseline value.

Morimoto et al. (1984) have reported that the lymphocytes of passive smokers are more sensitive to the induction of SCE by mitomycin C treatment than the lymphocytes from non-exposed non-smokers. On the other hand, no difference in baseline levels of SCEs between the exposure groups was observed.

Various biochemical intake measures have shown that the passively exposed smokers are exposed to a significantly greater extent to tobacco-smoke constituents than the control non-smokers (Jarvis et al., 1984; 1985). For the present passive smokers, the concentrations of plasma thiocyanate

as well as the tobacco-specific plasma and urinary cotinine were increased as compared with the non-exposed controls (Husgafvel-Pursiainen et al., 1986a). In addition, the air-samples taken from the restaurants where the passive smokers worked were found to induce mutations in bacterial tests and SCEs in cell cultures (Husgafvel-Pursiainen et al., 1986b). Thus the exposure status of the passively exposed subjects is well documented.

In the present study, the smokers had, as a group, the highest mean value of both SCE and RI. The individual mean SCE/cell frequencies were not, however, dependent on RI suggesting that proliferation rate of the cells in culture does not influence SCE frequency. A similar lack of correlation was recently found by Dewdney et al. (1986) in a SCE study of 106 subjects, by Speit et al. (1986) in repeated cultures, and by Parkes et al. (1985). This is in contrast to the findings of a negative correlation by Lindblad and Lambert (1981) and by Lamberti et al. (1983) showing that slowly proliferating lymphocytes have higher SCE values, as suggested also by Snope and Rary (1979). A positive correlation between a statistical transformation of SCE/cell and RI was reported by Wulf et al. (1986).

By examining 3 groups of individuals with different degrees of tobacco-smoke exposure, this study did not find an influence of passive exposure on mean SCE level or number of HFCs in 20 waiters and waitresses. The cigarette smokers in the study did, however, show the highest mean SCE and RI as well as the highest number of HFCs as a group. The negative result of the present study suggests a need for a larger study population to detect this type of low-level exposure.

Acknowledgements

I wish to thank Ms. Hilkka Järventaus for technical assistance; Ms. Ritva Luukkonen, M.Sc., for help in statistical analyses; and the restaurant personnel who participated in the study. The work was financially supported by a grant from the Finnish Medical Board of Health (grant No. 3.2.5/85).

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Communicated by R.J. Preston

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of voluntary self-regulation, and demonstrates how inadequate such an approach is to the control of pharmaceutical advertising in medical journals. The only sanction that can be applied against an advertisement that violates the PAAAB's code is to insist on modification (or withdrawal) of the advertisement. There is no requirement for the company to inform doctors and others of mistakes in an advertisement as submitted. Nor does the PAAAB make public the results of complaints against advertisements even when those complaints have been upheld.

The PAAAB and its code gives doctors, pharmacists, and the public a false sense of security. Godden refers to the "serious ethical standards" incorporated in the code but major aspects of advertisements are neglected and the provisions are so vague that one can find numerous examples of misleading advertisements in Canadian medical journals.

The code prohibits advertising which is prejudicial to either sex but this does not stop companies from using pictures of naked women to illustrate their advertisements. In an advertisement for one dermatological product a profile of a crouching naked woman was accompanied by the highly suggestive caption "For a great performance". Sexual stereotyping does not seem to be covered by the code: when the female schizophrenic is "under control" with pimozide ("Omp") she is at a children's birthday party, while the "uncontrolled" male schizophrenic is at work on a construction site.

According to the code "any advertising containing therapeutic claims... must include a complete reference listing". However, in an advertisement for alprazolam ("Xanax") Upjohn promised that its product had "outstanding therapeutic effectiveness" in patients with anxiety and yielded a "lower incidence of drowsiness, depression and confusion". There were no references to judge the drug's "outstanding" efficacy nor did the advertisement mention with what the incidence of side-effects was being compared. Even when references are provided they do not always seem to be "the most recent available and... consistent with current medical opinion", as the code requires. An advertisement for "Tennant" oral rinse contained eleven references—one came from an English language publication, one from a German journal, and the remaining nine were based on "data on file" at Riker and seemingly of the testimonial type.

"The main advertising message and the prescribing information" are supposed to be either "adjoining, or clearly page referenced" but the code does not say anything about the message in these two parts being contradictory. An advertisement for clonidine ("Capegrip") was headlined "Lower his blood pressure... not his potency" but on the next page in the free print under adverse effects appeared "impotence".

These examples show that the PAAAB and its Code of Advertising Acceptance are a smokescreen that the drug industry uses to continue to print misleading advertising. Unless the WHO or the Medicines Commission wish to condone such practices the PAAAB is not a model that they should look to.

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JOEL LEXCHON

USELESS DRUGS ARE NOT PLACEBOS

Sir,—Dr Brand (Nov 1, p 1034), commenting on our paper on entrapmental disorders caused by clonidine and flunarizine (Oct 11, p 853), recommends clonidine for motion sickness, but he does not cite any randomised placebo-controlled trials. The dose of clonidine originally recommended for this indication was 7.5 mg; most presentations of the drug today contain 75 mg.

Dr Osterweil (Nov 22, p 1218) cites three "well-designed, placebo-controlled trials" of clonidine and flunarizine for vertigo and dizziness. The first has not been uncovered even by the British Library Document Supply Centre. In one study, although significant effects on the frequency and duration of vertigo were found, severity was not influenced by flunarizine.¹ In the other study, the severity of attacks, measured on a 20-point scale, improved 3.15 points in the flunarizine-placebo group and 1.74 points in the placebo-flunarizine group.² Another double-blind trial could not demonstrate the superiority of flunarizine over placebo because of "further improvement in two placebo-treated patients".³

These studies show that the benefits, if any, of flunarizine in the treatment of vertigo are marginal.

Dr Krupp and Dr Loew (Dec 6, p 1335) cite J. R. Wizenborn's review of fifteen placebo-controlled trials of co-dergocrine in senile dementia. Although statistically significant differences are reflected in clinical significance is not mentioned. F. Reubi's review, also cited by Krupp and Loew, concludes that improvement in subject variables is usually less than one point on a seven-point scale. Most trials do not exceed 12 weeks, and three long-term trials have reported significant differences between drug and placebo only at specific time periods—after 24 (but not at 12) weeks,⁴ after 12 weeks (but not later),⁵ and after 15 (but not at 6) months⁶ (in which 154 of the patients dropped out during the study). A 1982 review on thirty-eight trials with co-dergocrine included less than 170 outpatients randomised to the drug.⁷ In eight of the twenty-six studies appraising global change, significant improvement with co-dergocrine was not demonstrated. The possibility that other negative results may have not been published should also be considered. Despite its disputed efficacy, co-dergocrine is the eleventh most widely prescribed drug in the world.⁸ This may reflect the lack of effective treatments for senile dementia, but it does not support the value of co-dergocrine.

Dr Moore and colleagues (Nov 22, p 1217) urge "better education of the public that drugs will not cure everything under the sun". We agree, however, the responsibility for this education falls mainly on the prescriber. The pressure from patients is pressure not necessarily to prescribe but for some minutes of communication, information, and education about symptoms and their treatment. In 1977, 1.4 prescriptions of co-dergocrine per 1000 population were filled in the UK; in other countries the corresponding figures were 4.3 in the USA, 3.3 in Germany, 5.5 in France, 88 in Italy, and 203 in Belgium⁹ (the 1983 figure for Spain was 158). We do not think that these differences are due to different education of the public in these countries.

Since our publication, on 16 patients who had entrapmental disorders associated with treatment with clonidine and flunarizine, the Spanish Centre for Adverse Drug Reactions Monitoring has received 49 further such reports. 20 who have been followed up recovered 2 weeks to 6 months after stopping medication. At least 7 had had pertussis for a year or more before their illness was diagnosed as drug induced.

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PASSIVE SMOKING IN ADOLESCENTS: ONE-YEAR STABILITY OF EXPOSURE IN THE HOME

Sir,—Cocaine levels in body fluids can detect non-smokers' current exposure to other people's smoke,¹⁻⁴ but for evidence to be valuable for epidemiological investigations it is important for the concentration in a single sample to demonstrate exposure over a period which is meaningful in terms of health risks. We have studied saliva cocaine concentrations over one year in non-smoking schoolgirls aged 11-16.

The girls at an inner-city girls' comprehensive school in south London were surveyed in January to March, 1985, and again in January to March, 1986. They completed a questionnaire on

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UPTAKE OF SIDESTREAM SMOKE BY SYRIAN GOLDEN HAMSTERS*

(Sidestream smoke; nicotine; cotinine; Inhalation bioassay)

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(Received 10 September, 1986)

(Accepted 13 September, 1986)

SUMMARY

An inhalation bioassay with Syrian golden hamsters is being conducted to evaluate the toxic and carcinogenic potential of cigarette sidestream smoke (SS) relative to mainstream smoke (MS). A Hamburg II smoking machine is used to deliver MS by nose-only exposure to hamsters and a modification allows for the simultaneous collection of SS for whole-body delivery to a different rack of animals. The tolerated dose of SS was determined by varying the air/smoke dilutions drawn through the animal restrainers. Preliminary data indicated that 20% carboxyhemoglobin (COHb) could be obtained in SS-exposed animals without fatality. Optimum exposure levels were determined. Monthly measurements of COHb, nicotine and cotinine indicate that the SS-exposed animals are absorbing slightly higher amounts of these smoke constituents than the MS-exposed hamsters. Tumor incidence and carcinogenicity data are being collected through complete necropsy and histology protocols and uptake data continue to be collected. These studies should help elucidate the carcinogenic potential of SS which has been suggested from its composition and from recent epidemiological data of cancer incidence in non-smokers.

INTRODUCTION

Sidestream smoke (SS) of tobacco products is a major pollutant of indoor atmospheres. Available knowledge on its formation, chemical composition, and fate in the environment [1-4], as well as clinical observations on selected population groups, have raised questions about the risk for development of chronic diseases

* Presented at the International Experimental Toxicology Symposium on Passive Smoking, October 23-25, 1986, Essen (F.R.G.).

Abbreviations: COHb, carboxyhemoglobin; MS, mainstream smoke; SS, sidestream smoke.

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following exposure of non-smokers to environmental tobacco smoke (ETS). Early studies have emphasized subjective annoyance and possible allergic reactions of persons exposed to ETS [5, 6] while recent investigations have evaluated the possible correlation between ETS and an increased risk for cancer development in non-smokers. Several studies point to a positive correlation between ETS exposure and cancer risk [7-9] while other data bases have not confirmed these findings [9-10].

While the toxicology and carcinogenicity of mainstream smoke (MS) have been studied extensively [14-15], investigations into the carcinogenic potential of SS in animals are needed. We present here comparative data on the uptake of tobacco smoke constituents by Syrian golden hamsters exposed to either MS or SS in a specially designed system.

MATERIALS AND METHODS

Sidestream smoke collection and delivery

In MS inhalation assays, 10 Syrian golden hamsters received the mainstream smoke of 30 cigarettes generated in the Hamburg II smoking machine and diluted with air at a ratio of 1:7 twice daily. Such exposures over several months have produced reproducible carcinogenic changes in several studies [16, 17].

For SS exposure a 40-cm³ collection chamber made of plexiglass was mounted above the rotating head of the Hamburg II machine. The collected SS was drawn through a baffle assembly and delivered via tygon tubing to the hamsters who resided in plexiglass tubes identical to those used for MS exposure. However, MS exposure is nose-only, so that uptake is only by inhalation while the SS exposure system draws smoke through each tube, exposing the animal by both inhalation and physical contact. The exit of each exposure tube is connected to a vacuum manifold such that the flow rate can be regulated.

The dose of SS delivered to the animals and the air dilution can be adjusted by varying the height of the collection chamber from the burning cones of the cigarettes and by adjusting the speed of the pump.

Mainstream and sidestream smoke exposure protocol

Preliminary experiments were conducted to establish the dose of SS which would be tolerated by the animals. The hamsters were then gradually exposed to MS or SS over a 6-week period. The full exposure regimen was carried out twice each day for a total of 24 months. Animals which fell ill during the exposure period were killed and necropsied. At the completion of the experiment, all animals were killed and necropsied [17].

Total particulate matter and nicotine delivery

Total particulate matter (TPM) and nicotine were trapped on Cambridge filter pads placed at the exit of the SS exposure tubes. To ensure that nicotine was trapped

from both gas phase and particulate phase, the filters were pre-treated with 0.01 N potassium bisulfate. The TPM trapped on the filter pads during a single round of smoke exposure was determined gravimetrically; nicotine was quantitated by gas chromatography after placing the Cambridge filters in 5 ml methanol basified with 0.1 N sodium hydroxide.

Uptake of tobacco smoke components

During the experimental protocol, animals were tested each month to assess the uptake of nicotine and COHb by the treatment vs. control groups. Blood was collected from the orbital sinus of 3 animals in each group. Different animals were tested each month to minimize stress induced by repeated eye bleeding.

Nicotine and cotinine were quantitated in plasma by a modified radioimmunoassay (RIA) [18-20]. Coefficients of variation for these assays were 6% with sensitivity of accurate measurements set at 1 ng/ml.

% COHb was determined spectrophotometrically with an IL-282 CO-oximeter (Instrumentation Laboratories). Calibration with certified standards and quality control yielded reliable analyses with a daily variation of less than 1%.

RESULTS

The highest concentration of sidestream smoke tested was that generated by 30 cigarettes and diluted with 10 l/min of air. This exposure was not well tolerated; animals exhibited loss of balance and had COHb levels averaging 28%. Larger dilutions provided tolerable COHb levels and acceptable indications of nicotine uptake (Table I).

On the basis of the preliminary tests it was decided to acclimatize the animals to SS at a dilution of 20 l/min over a 6-week period. The hamsters maintained body weight or grew during the first 8 months of this bioassay. No differences were observed between the exposed groups and controls.

The measurements of TPM and nicotine delivered in each round of exposure to an individual hamster are given in Table II. The SS data are averaged from nine

TABLE I
EFFECT OF CONCENTRATION OF SIDESTREAM SMOKE

Air dilution of SS	COHb (%)	Nicotine (ng/ml)	Animals (N)
10 l/min	28.2	90.0*	5
13 l/min	15.1	14.0	4
15 l/min	13.4	1.6	3

* Taken immediately following a single exposure to SS. Cotinine was not detected immediately following exposure.

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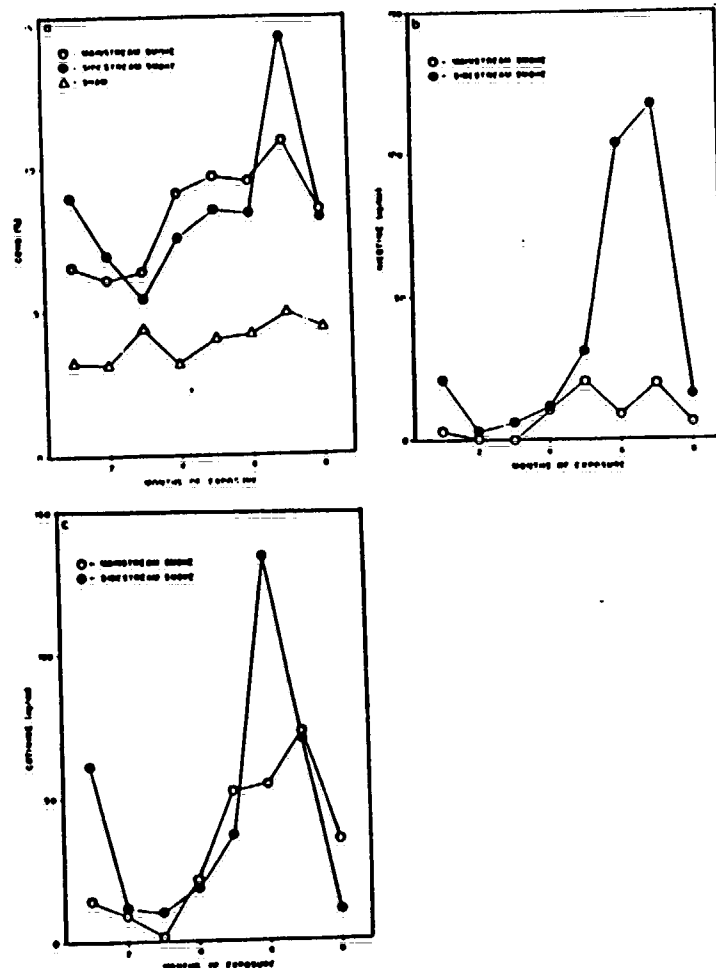


Fig. 1. (a) Blood levels of COHb in Syrian golden hamsters exposed to MS or SS as well as sham-treated animals during the first 8 months of an inhalation protocol. (b) Plasma nicotine levels in Syrian golden hamsters exposed to MS or SS. Sham-treated animals did not have detectable levels of nicotine. (c) Plasma cotinine levels in Syrian golden hamsters exposed to MS or SS. Sham-treated animals did not have detectable levels of cotinine.

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TABLE II

TPM AND NICOTINE AS DELIVERED IN ONE ROUND OF EXPOSURE

Source of smoke	TPM (mg/hamster)	Nicotine (mg/hamster)
MS	15.7	1.05
SS	36.7 \pm 9.9%	1.87 \pm 10.21

* Mean value of 9 determinations \pm SD (%)

determinations and the MS data are calculated from the delivery and dilution of smoke from 30 2R1 cigarettes smoked on the Hamburg II smoking machine.

Uptake of carbon monoxide and nicotine was measured monthly. Fig. 1a illustrates average COHb levels observed in the exposed group and the sham-treated controls. Ocular bleeding was conducted approx. 1 h following exposure, which leads to large fluctuations in nicotine levels, since the biological half-life of this constituent is less than 1 h [19]. Due to its longer half-life, the major nicotine metabolite, cotinine, is a more reliable marker of nicotine uptake, as shown in Fig. 1b and c.

ACKNOWLEDGEMENT

This study was supported by Grant No. CA-29580 from the U.S. National Cancer Institute.

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TXL 01704

THE EFFECT OF SMOKE AGE AND DILUTION ON THE CYTOTOXICITY OF SIDESTREAM (PASSIVE) SMOKE

(L-929 cells; cigarette design; cell death)

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(Received 3 September, 1986)

(Accepted 15 September, 1986)

SUMMARY

Decreases in the mortality of monolayer cultures of L-929 cells exposed to sidestream (passive) smoke with increases in smoke age and dilution have been reported. In the current study, the concentration of sidestream smoke (SS) to which cells were exposed was directly correlated with % mortality ($r = 0.987$) having 0% mortality at a concentration of 1.19% and a calculated mortality of 95.62% at a concentration of 100%. The \ln of % mortality was correlated with increases in smoke age ($r = -0.9999$) and the regression equation was used to calculate 0 mortality at an age of 30 s and 393% mortality at the time of smoke generation. In addition, when sidestream smoke generated from a low-yield, filtered, modern design experimental cigarette was compared with that generated from a high-tar non-filtered reference cigarette, a lower number of puffs of smoke from the low-yield cigarette than from the high tar cigarette was necessary to yield 50% mortality of cells.

INTRODUCTION

A peristaltic pump smoke exposure system [1] has been used to determine the cytotoxic effects of SS smoke (passive smoke) on monolayers of murine L-929 cells [2]. The results of these studies indicated that a dose response for cytotoxicity of SS smoke existed, and that cytotoxicity of smoke decreased with increased dilution or aging of the smoke.

In a study of the effects of SS flow rates on smoke concentrations and yield of

* Presented at the International Experimental Toxicology Symposium on Passive Smoking, October 23-25, 1986, Essen (F.R.G.).

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INDOOR AIR '87

**Proceedings of
The 4th International Conference on
Indoor Air Quality and Climate
Berlin (West), 17-21 August 1987**

2

**Volume 2
Environmental Tobacco Smoke, Multicomponent Studies,
Radon, Sick Buildings, Odours and Irritants,
Hyperreactivities and Allergies**

Institute for Water, Soil and Air Hygiene

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SIDESTREAM SMOKE UPTAKE BY SYRIAN GOLDEN HAMSTERS
IN AN INHALATION BIOASSAY

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Abstract

An inhalation bioassay with Syrian golden hamsters is being conducted to evaluate the toxic and carcinogenic potential of sidestream smoke (SS) relative to mainstream smoke (MS) of the same cigarette. This long-term bioassay utilizes dilutions of smoke which do not cause acute toxicity in the animal. In each exposure, the SS instrument delivers 37 mg of tar and 1.8 mg nicotine to each animal while MS nose only delivery provides 16 mg tar and 1.1 mg nicotine. Monthly measurements of carboxyhemoglobin (COHb), nicotine and cotinine have shown that SS and MS exposed animals are absorbing tobacco smoke constituents. Survival data collected after 18 months of the bioassay indicate a higher mortality for SS exposed animals relative to MS exposed hamsters.

Introduction

Sidestream smoke (SS) is that fraction of tobacco smoke which is emitted from the burning end of a cigarette between puffs and constitutes a major pollutant of indoor air (1,2). Its chemical composition and environmental distribution have raised questions about the risk for development of disease among exposed persons. Several studies suggest a correlation between exposure to other people's smoking behavior and cancer risk (3,5) while other researchers have not confirmed this association (6).

Inhalation studies on the carcinogenicity of MS have been conducted on a variety of laboratory animals (7,8) while inhalation studies with SS are limited. We present here data on a comparison study of MS and SS inhalation with Syrian golden hamsters.

Materials and Methods

Delivery of SS and MS to Animals

The SS collection and delivery system used for this bioassay have been described previously (9).

Briefly, the system utilizes a 40 cm² collection hood which is mounted over the rotating head of a Hamburg II smoking machine. The diluted SS is drawn through restraining tubes containing the animals and diluted MS is delivered by nose only exposure to another set of hamsters. The dose is adjusted by varying the dilution of the smoke before delivery to the animals.

Animals are exposed twice daily, seven days each week. During each round of smoking, approximately 37 mg of total particulate matter and 1.9 mg of nicotine are delivered to the SS exposed animals while the animals receiving MS are exposed to approximately 16 mg tar and 1.1 mg nicotine.

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Sixty animals began the protocol in the SS exposed group with an equal number receiving MS. Sham controls were utilized for both modes of inhalation.

Uptake of Tobacco Smoke

Animals were tested each month to assess the uptake of nicotine and COHb. Blood collected from the orbital sinus of 3 animals in each group was quantitated for % COHb, nicotine and cotinine levels. COHb was determined spectrophotometrically with an IL-282 Co-oximeter (Instrumentation Laboratories). Nicotine and cotinine were quantitated by radioimmunoassay (RIA) (10,11).

Results

Preliminary tests determined the optimum dilution for SS delivery to be 13 l/min, a level which was tolerated by the animals and presented minimal acute toxic effects. The bioassay began with an acclimatization phase during which animals were gradually exposed to increasing amounts of SS or MS and sham treated animals were held in clean instruments for increasing amounts of time.

The average uptake of CO and nicotine is reflected in percent COHb as well as the amount of cotinine present in plasma. The levels found in MS or SS exposed animals are shown in Figure 1 and 2 and reflect levels seen over 16 months of the protocol.

The SS exposed animals received whole body exposure while the MS exposure was nose-only. Sham treated animals did not have detectable levels of nicotine or cotinine and maintained low COHb levels throughout the bioassay. The variability of results across time can reflect metabolic differences in different animals selected each month for ocular bleeding or actual differences in uptake by different animals at various times of the year. These animals do exhibit lower respiratory rates during the winter months and maintain a natural biorhythm despite the temperature and light controlled environment in which they are housed.

Table I contains the survival data for animals in each exposure group and for pooled sham treated controls. The sentinel or cage controls as well as those animals receiving either method of sham treatment declined in number more rapidly than the smoke exposed animals. Such survival data have been seen in previous animal studies with MS inhalation (8,12).

Animals exposed to SS decreased in numbers more rapidly than their MS exposed counterparts. The largest numbers of deaths occurred in the 14-15 months of the bioassay. In both groups, more males than females have survived with only 40% of MS exposed females and 30% of SS exposed females remaining after 15 months.

Body weight for surviving animals has remained consistent across the smoke exposed groups and lower than both groups receiving sham exposure. Figure 3 illustrates the growth curves of these animals with males and females placed together, although the males were consistently 10% heavier. The weight changes over time showed similar responses for both sexes. The growth and weight gain of exposed hamsters was very similar for both MS and

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SS exposed groups. The sham exposed and cage control animals gained more weight during the early months of the protocol and had higher mortality rates during the first year of the bioassay.

Table 1: Percentage of animals in each group surviving the bioassay (a)

Months	Sham Treated (b)	Mainstream Exposed	Sidestream Exposed
3	98	100	91
6	88	97	85
9	80	93	84
12	41	76	70
15	23	55	33

a Each exposure group began with 30 males and 30 females while sham treated groups had 20 animals for each sham protocol.

b Survival data for MS-sham treated and SS-sham treated have been pooled.

Discussion

Inhalation bioassays with Syrian golden hamsters have demonstrated the carcinogenicity of MS in this animal species. Since the hamster is more resistant to respiratory infections than are rats and mice and is also more tolerant to the acute effects of tobacco smoke, it is, at present, the best rodent model available for inhalation studies to compare MS and SS carcinogenicity.

Our ongoing bioassay has demonstrated that animals exposed to MS or SS absorb carbon monoxide and nicotine and exhibit variable levels of COHb and the nicotine metabolite, cotinine. Carboxyhemoglobin levels averaged 7-8% for both groups, a percentage often seen in human cigarette smokers (12). Cotinine averaged between 40 - 50 ng/ml, a level lower than that observed in the majority of human smokers. This is consistent with the low level of breathing done by these restrained animals and contrasts with the deep inhalation patterns of active cigarette smokers.

Survival and weight data thus far indicate that smoke exposed animals live longer and do not grow as large as sham treated or cage controls. This is consistent with previous findings (2) on Syrian golden hamsters.

The bioassay will continue for a total of 18 months of MS or SS exposure. A complete necropsy of each animal will be conducted to compare tumor incidence across the groups exposed to MS or SS as well as to sham treated animals. Histopathological evaluation of tumors from animals in each exposure will be presented. Hopefully, these studies will provide a basis for comparison of the carcinogenic potential of MS and SS.

These studies were supported by a grant from the National Cancer Institute CA 29580. The authors gratefully acknowledge the excellent technical assistance of Dorothy Chary and John Bevilacqua.

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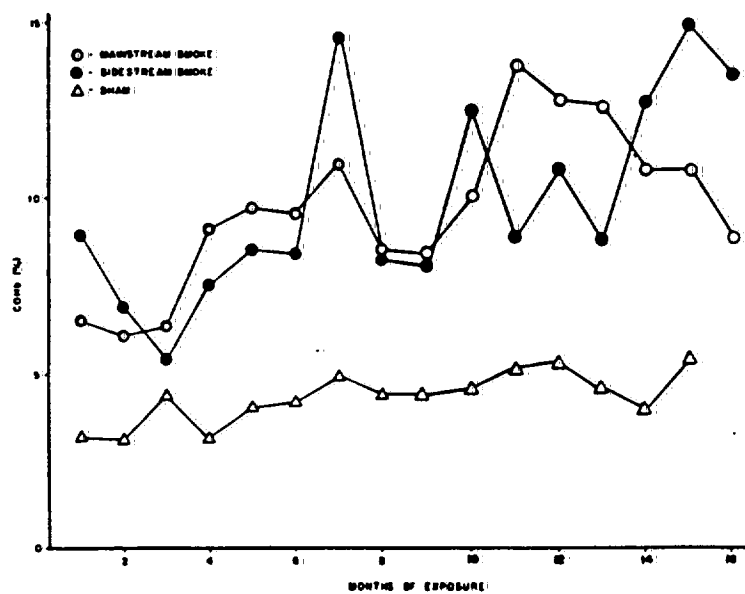


Figure 1 - Uptake of COHb by hamsters exposed to mainstream or sidestream smoke over 16 months.

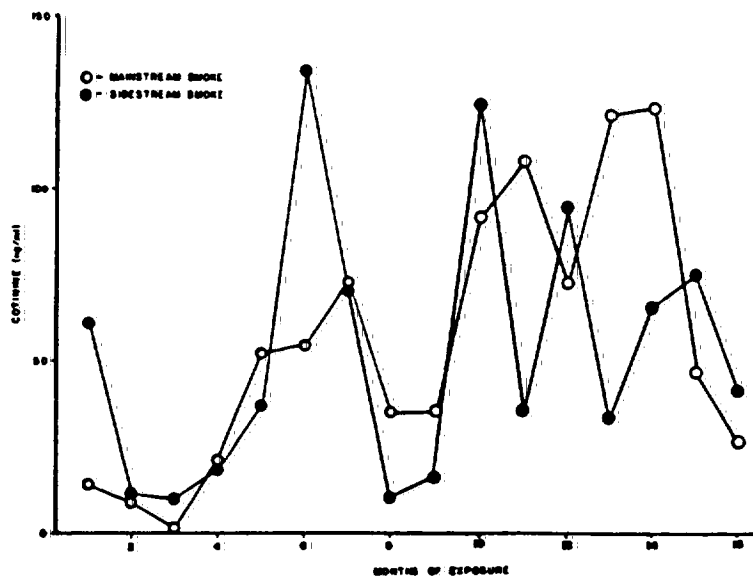


Figure 2 - Plasma levels of cotinine in hamsters exposed to mainstream or sidestream smoke over 16 months.

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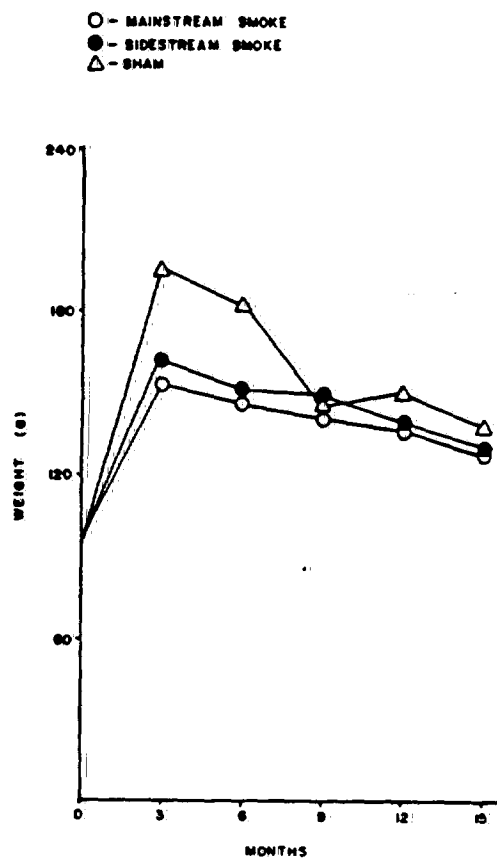


Figure 3 - Weight gain by hamsters exposed to mainstream or sidestream smoke over 15 months.

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DEPARTMENT OF HEALTH AND HUMAN SERVICES
PUBLIC HEALTH SERVICE

MAY 17 1988

APPLICATION
FOR CONTINUATION GRANTREVIEW GROUP TYPE ACTIVITY GRANT NUMBER (insert on all pages)
SEC - I
(15) 5 P01 CA29580-02

TOTAL PROJECT PERIOD

From: 06/15/81 Through: 05/31/92

REQUESTED BUDGET PERIOD

From: 06/01/88 Through: 05/31/89

To be verified by applicant. Check information in items 1 through 6. If incorrect, furnish correct information in item 13.

1. TITLE OF PROJECT

EXPERIMENTAL TOBACCO CARCINOGENESIS

2a. PRINCIPAL INVESTIGATOR OR PROGRAM DIRECTOR
(name and address, street, city, state, zip code)HOPPMANN, DIETRICH
AMERICAN HEALTH FOUNDATION
320 EAST 43RD STREET
NEW YORK, NY 10017

4. APPLICANT ORGANIZATION (name and address, street, city, state, zip code)

AMERICAN HEALTH FOUNDATION
320 EAST 43RD STREET
NEW YORK, NY 10017

5. ENTITY IDENTIFICATION NUMBER

1132552627A1

2b. DEPARTMENT, SERVICE, LABORATORY OR EQUIVALENT
ENVIRONMENTAL CARCINOGENESIS

2c. MAJOR SUBDIVISION

6. TITLE AND ADDRESS OF OFFICIAL IN BUSINESS OFFICE OF APPLICANT ORGANIZATION

DAVID D. RUBY
AMERICAN HEALTH FOUNDATION
ONE DANA ROAD
VALHALLA, NY 10595

3. ORGANIZATIONAL COMPONENT TO RECEIVE CREDIT FOR BIOMEDICAL RESEARCH SUPPORT GRANT (see instructions)

60 OTHER RESEARCH ORGANIZATION

Complete the following (see instructions)

7. HUMAN SUBJECTS

7a. ☐ NO ☒ YES☐ Exemption # _____OR
☒ IRB Approval Date 04/28/88

7b. Assurance of Compliance # M1193-01

8. VERTEBRATE ANIMALS

8a. ☐ NO ☒ YES IACUC Approval Date 4/16/88

8b. Animal Welfare Assurance # A3293-01

9. PERFORMANCE SITE(S) (organizations and addresses)

American Health Foundation
One Dana Road
Valhalla, NY 10595

10. COSTS REQUESTED FOR BUDGET PERIOD

10a. DIRECT \$558,767 10b. TOTAL \$ 977,842

11. INVENTIONS (see instructions)

☒ NO☐ YES☐ Previously reportedOR
☐ Not previously reported

TELEPHONE INFORMATION

12a. PRINCIPAL INVESTIGATOR
OR
PROGRAM DIRECTOR (Item 2a)AREA
CODETELEPHONE NO.
AND EXTENSION

914

592-2600

12b. NAME OF BUSINESS OFFICIAL
(Item 6)

David D. Ruby

914

592-2600 x305

12c. NAME AND TITLE OF OFFICIAL
SIGNING FOR APPLICANT
ORGANIZATION (Item 15)

David D. Ruby

914

592-2600 x305

13. USE THIS SPACE FOR CORRECTIONS TO ITEMS 1 THROUGH 6. INDICATE THE NUMBER(S) WHERE ANSWERS APPLY.

14. PRINCIPAL INVESTIGATOR/PROGRAM DIRECTOR ASSURANCE: I agree to accept responsibility for the scientific conduct of the project and to provide the required progress reports if a grant is awarded as a result of this application. Willful provision of false information is a criminal offense. (U.S. Code, Title 18, Section 1001.)

SIGNATURE OF PERSON NAMED IN 2a
(In ink. "Per" signature not acceptable)

DATE

04/29/88

15. CERTIFICATION AND ACCEPTANCE: I certify that the statements herein are true and complete to the best of my knowledge, and accept the obligation to comply with the Public Health Service terms and conditions if a grant is awarded as the result of this application. A willfully false certification is a criminal offense. (U.S. Code, Title 18, Section 1001.)

SIGNATURE OF PERSON NAMED IN 12c:
(In ink. "Per" signature not acceptable)

DATE

04/25/88

PHS 2590 Optional (Rev. 9/84)

PAGE 1

RETURN COMPLETED APPLICATION TO PHS AS SOON AS POSSIBLE:
NO LATER THAN 1 APRIL 1988

2023381387

REQUESTED BUDGET FOR NEXT BUDGET PERIOD		FROM 5/1/88	THROUGH 4/30/89	GRANT NUMBER 5 P01 CA29580-08	
Follow instructions carefully.					
A. ITEMIZE DIRECT COSTS REQUESTED FOR NEXT BUDGET PERIOD		1 TYPE APPT	2 % OF APPT	3 EFF ON PROJ	DOLLAR AMOUNT REQUESTED
PERSONNEL <small>(Applicant organization only)</small>					Comments
NAME	ROLE IN PROJECT				SALARY FRINGE BENEFITS TOTALS
Stephen S. Hecht, Ph.D.	Subproject #1				
Klaus D. Brunnemann, M.S.	Subproject #2				
Bogdan C. Prokopczyk, Ph.D.	Subproject #3				
Sharon Murphy, Ph.D.	Subproject #5				
Nancy J. Haley, Ph.D.	Subproject #6				
Dietrich Hoffmann, Ph.D.	Core Component A				
Carol A. Meschter, DVM., Ph.D.	Core Component B				
Abraham B. Rivenson, M.D.	Core Component C				
SUBTOTALS					351,407 65,008 416,416
CONSULTANT COSTS					
EQUIPMENT					
SUPPLIES					
Subproject #1	31,820	Core Component A	10,300		
Subproject #2	19,820	Core Component C	2,800		
Subproject #3	14,390				
Subproject #5	11,510				
Subproject #6	10,199				
					100,639
TRAVEL		DOMESTIC \$1,040 x 7			7,280
		FOREIGN			
PATIENT CARE COSTS		INPATIENT			
		OUTPATIENT			
ALTERATIONS AND RENOVATIONS					
CONSORTIUM/CONTRACTUAL COSTS					
OTHER EXPENSES		Subproject #6 3,610			
Subproject #1	1,800	Core Component A	9,500		
Subproject #2	11,012	Core Component C	1,800		
Subproject #3	600				
Subproject #5	6,310				
					34,432
TOTAL DIRECT COST		(Enter on Page 1, Item 10a)			\$ 556,767

2023381388

REQUESTED BUDGET FOR: NEXT BUDGET PERIOD Follow instructions carefully		FROM: 5-1-88	THROUGH 4/30/89	GRANT NUMBER 5 P01 CA29580-08 Subproject #6		
A. ITEMIZE DIRECT COSTS REQUESTED FOR NEXT BUDGET PERIOD		1	2	3	DOLLAR AMOUNT REQUESTED	
PERSONNEL (Applicant organization only)		TYPE	% OF	EFF ON	COMMITMENTS	
NAME	ROLE IN PROJECT	ADPT	ADPT	PROJ	SALARY	FRINGE BENEFITS
Nancy J. Haley, Ph.D.	Principal Investigator	1.00	20%	0.20	[1]	
Lisa Blear, B.S.	Res Assistant	1.00	100%	1.00		
Stephen Colosimo, M.S.	Asst Res Scient	1.00	80%	0.80		
[1] Supported on CCSG CA17613						
SUBTOTALS					41,603	7,897
CONSULTANT COSTS						
EQUIPMENT						
SUPPLIES						
Chemicals	1,200	Animal purchase	710			
Radioactive Chemicals	1,980	Animal maintenance	1,930			
Chromatography supplies	490	Animal supplies	130			
Solvents, reagents & gases	1,289					
Gloves & syringes	740					
Glassware & labware	990			10,199		
Safetyware & protect. cloth.	740					
TRAVEL	DOMESTIC	PI to Clinical Chemists Conference			1,040	
	FOREIGN					
PATIENT CARE COSTS	INPATIENT					
	OUTPATIENT					
ALTERATIONS AND RENOVATIONS						
CONSORTIUM/CONTRACTUAL COSTS						
OTHER EXPENSES						
Graphics & illustrations	395	Volunteer fees	1,000			
Page charges & processing fee	620					
Reprints	495			3,610		
Histology slides	1,100					
TOTAL DIRECT COST (Enter on Page 1, Item 10a)					\$	54,149

REPLACEMENT OF
EXPLANATION OF

Subproject 1

Shashi Sharma, MS, replaced J. Rubin.
Ronit Zilberbolm replaced I. Veals whose effort was 100%
Ronit Zilberbolm's effort is 70%

Subproject 2

Mirjan Djordjevic, Ph.D. replaced R. Onrust at 50% due to
funding plan restrictions.
Colleen Armlin replaced L. Genoble.

Subproject 3

Stayed the same.

Subproject 4

No longer exists.

Subproject 5

Sharon Murphy, Ph.D. replaced Ed LaVoie and John Adams. Dr.
Murphy will be 25%.
P. Tucciarone replaced Nalband Hussain.
M. Defloria replaced M. Djordjevic whose effort was 25%
M. Defloria's effort is 50%.

Subproject 6

Lisa Bier replaced J. Connolly
Nancy Haley will be supported by Core CA17613.

Core Component A

Sharon Murphy replaced E. LaVoie. Dr. Murphy will be 5%.
Karl Aebig replaced S. Colosimo
Jonathan Cox replaces replaces K. O'Mara-Adams.
Ireana Liutkus replaced D. Conroy

Core Component B

Carol Meschter replaces A. Rivenson.
Rotating Staff was 55 hours. The hours are now 40.8 or
117%.

Core Component C

Shigeyeyriki Sugie replaced by H. Maruyma, MD.

2023381390

EXPERIMENTAL TOBACCO CARCINOGENESIS 2 PO1 CA29580-07

SUBPROJECT 6: SIDESTREAM SMOKE CARCINOGENESIS

PROGRESS REPORT

Period covered June 1, 1987 - April 1, 1988

Nancy Jean Haley, Ph.D.	Associate Chief Division of Nutrition and Endocrinology	20%
Stephen G. Colosimo, M.S.	Senior Research Biochemist	100%
J.D. Adams, M.S.	Section Head Division of Environmental Carcinogenesis	
K.D. Brunneemann, B.S.	Section Head Division of Environmental Carcinogenesis	

I. PUBLICATIONS

Sepkovic, D.W. and Haley, N.J.
Metabolism of nicotine in smokers and nonsmokers. In: Tobacco Smoking and Nicotine (W.R. Martin, G.R. Van Loon, E.T. Iwamoto and L. Davis, eds.). Plenum Publishing Corp. 1987, pp. 375-388.

Haley, N.J., Adams, J.D., Alzofon, J. and Hoffmann, D.
Uptake of sidestream smoke by Syrian golden hamsters. Toxicology Lett. 35: 83-88, 1987.

Haley, N.J., Adams, J.D., Axelrad, C.M. and Hoffmann, D.
Sidestream smoke uptake by Syrian golden hamsters in an inhalation bioassay. Indoor Air '87, vol. 2, 1987; pp. 68-73.

Hoffmann, D., Brunneemann, K.D., Haley, N.J., Sepkovic, D.W. and Adams, J.D.
Nicotine uptake by nonsmokers exposed to passive smoking under controlled conditions and the elimination of cotinine. Indoor Air '87, vol. 2, 1987; pp. 13-17.

Goldstein, G.M., Collier, A., Etzel, R., Lewtas, J. and Haley, N.J.
Elimination of urinary cotinine in children exposed to known levels of sidestream cigarette smoke. Indoor Air '87, vol. 2, 1987; pp. 61-67.

Haley, N.J. and O'Neill, I.K.
Collection of urine for prospective studies in passive smoking.
In: Environmental Carcinogens: Methods of Analysis and Exposure Measurement, Vol. 9, Lyon, France 1987; pp. 293-297.

Henderson, F.W., Morris, R., Read, H.F., Hu, P.C., Mumford, J.L., Forehand, L., Burton, R., Lewtas, J., Hammond, S.K. and Haley, N.J.

Serum and urine cotinine as quantitative measures of passive tobacco smoke and exposure in young children. Indoor Air '87, vol. 2, 1987; pp. 18-21.

Hoffmann, D., Wynder, E.L., Hecht, S.S., Brunnemann, K.D., LaVoie, E.J. and Haley, N.J.

Chemical Carcinogens in Tobacco. In: Cancer Risks, Strategies for Elimination, (P. Bannasch, ed.). Springer Verlag, Germany, 1987; pp. 101-113.

Schiffman, M.H., Haley, N.J., Felton, J.S., Andrews, A.W., Koslow, A., Lancaster, W.P., Kurman, R.J., Brinton, L.A., Lonnem, L.B. and Hoffmann, D.

Biochemical epidemiology of cervical neoplasia: Measuring cigarette smoke constituents in the cervix. Can. Res. **47**: 3886-3888, 1987.

Haley, N.J., Sepkovic, D.W. and Hoffmann, D.
Elimination of cotinine from body fluids: Nicotine deposition in smokers, nonsmokers and chewers of nicotine gum. Am. J. Public Health (submitted).

LETTERS AND PRESENTATIONS

Sepkovic, D.W., Axelrad, C.M., Colosimo, S.G. and Haley, N.J.
Measuring tobacco smoke exposure: Clinical applications and passive smoking. 80th Annual Meeting and Exhibition of the Air Pollution Control Association, New York, NY 1987,

Sepkovic, D.W., Haley, N.J. and Hoffmann, D.
Urinary cotinine to estimate exposure to tobacco smoke (Letter) JAMA **25**: 1808, 1988.

Haley, N.J., Sepkovic, D.W., Louis, E. and Hoffmann, D.
Absorption and elimination of nicotine by smokers, nonsmokers, and chewers of nicotine gum. International Symposium on Nicotine, 10th International Congress of Pharmacology, Queensland, Australia, 1987.

Haley, N.J., Adams, J.D., Axelrad, C.M. and Hoffmann, D.
Sidestream smoke uptake by Syrian golden hamsters in an inhalation bioassay. Indoor Air '87, 4th International Conference on Indoor Air Quality and Climate, Berlin (West) Germany, 1987.

II. PROGRESS REPORT

The objectives of project 6 remain to evaluate the carcinogenicity of sidestream smoke relative to that of mainstream smoke in laboratory animals and to estimate the uptake of environmental tobacco smoke by smokers and nonsmokers.

A. Inhalation Bioassay

The first objective is being investigated in Syrian golden hamsters through long-term inhalation bioassays and short-term investigations of smoke constituent uptake and deposition. The inhalation bioassay was ended following 18 months of exposure to mainstream or sidestream smoke and animals surviving to this termination point were sacrificed.

The average level of carboxyhemoglobin (COHb) found in the animals was maintained between 6 and 9% over the 18 months of the study, a level similar to that seen in smokers (Figure 1). Cotinine levels over the same period were variable and generally lower than those found in smokers with COHb of 5-10% (Figure 2). Animals treated with sidestream smoke (SS) were exposed twice daily to higher amounts of tar and nicotine than mainstream smoke (MS)-exposed animals. Such differences in nicotine exposure were not seen in circulating cotinine levels. Both MS- and SS-treated animals weighed less than sham-treated animals (Figure 3), although the latter dropped weight near the end of the protocol as large numbers died with renal disease.

After 18 months, only 4% of sham-treated animals survived and the exposure protocol was terminated. A larger percentage of MS- and SS-exposed animals remained. This longer survival of smoke-exposed animals has been observed previously. Table I provides the survival data for this study. Histological evaluations, including step sections of the larynx and trachea were conducted on each animal in the study.

Table II presents the histopathology for the larynx and trachea from each group of animals. Overall, there was not a marked increase in tumor incidence in treated animals. Epithelial hyperplasia with small papillomas were noted only in exposed animals with 5 out of 60 animals presenting this type of lesion.

One animal exposed to MS had an adenoma of the lung and two of the SS-exposed animals had this lung tumor. None of the controls or sham-treated animals presented lung tumors.

Throughout the bioassay, biochemical indicators suggested that animals receiving MS or SS absorbed similar amounts of smoke constituents. At these doses, the incidence of tumors was fairly consistent between the groups.

B. Current Animal Studies

Work with human subjects has suggested a difference in nicotine metabolism or at least differences in cotinine elimination between smokers and nonsmokers. The inhalation bioassay described above demonstrated lower levels of cotinine in the blood of hamsters than would be expected based upon the dose provided and the carboxyhemoglobin level of the animals. We are, therefore, investigating the rate of nicotine metabolism in animals when they are first exposed to nicotine and following acclimatization to nicotine for 2 or 4 weeks. Additionally, possible inter-strain differences are being investigated by the use of 5 inbred strains of rats in the same protocol.

C. Human Exposure Studies

The elimination of nicotine from the bodies of smokers and exposed nonsmokers is a complex process which shows inter-individual variation. Earlier work has shown a longer cotinine residence time for passive smokers than for active smokers, a phenomenon which could be due to metabolic differences, differences in method of nicotine uptake or a concentration gradient effect.

To investigate these differences, 5 smokers quit tobacco use. Urine was collected for 5 days. They then used Nicorette gum for 3 days and continued urine collection. After 8 days, these ex-smokers and 5 never-smokers were exposed to sidestream smoke, twice daily for 2 days. Urine collection continued for 5 days past exposures. Each sample was analyzed by RIA for nicotine and cotinine and for thiocyanate. Urine creatinine was quantitated on a Kodak Ektachem 400. Terminal cotinine half-life ($t_{1/2}$) was calculated by the STRIPE program.

Urine cotinine elimination times are presented in Table 3 for each protocol. Data are presented for each individual subject as ng/ml urine and as ng/mg creatinine. Correlation coefficients and intercepts were determined using ng cotinine/mg creatinine derived data. Following cessation of smoking, subjects had a mean elimination time of 15.4 hrs. Concentration of cotinine in the urine did not appear to influence $t_{1/2}$ in these subjects. Both $t_{1/2}$ measures were highly correlated ($r=0.79$) and the means were similar (13.9 hrs. vs 15.4 hrs.).

Urine cotinine elimination times in subjects who used nicotine gum are given. Subject A was dropped for noncompliance and subject E for medical reasons not related to the protocol. Cotinine $t_{1/2}$ averaged 18.2 hrs. with creatinine and 17.7 hrs without it. Correlation between these measures was $r=0.99$. Ex-smokers exposed to sidestream smoke presented a mean $t_{1/2}$ of 27.5 hrs. for cotinine excreted per ml and a mean elimination time of 36.9 hrs. when normalized. The correlation was much more moderate ($r=0.60$). As indicated by the y-intercepts, urine concentrations averaged less than 30 ng/mg creatinine. In never-smokers exposed to sidestream smoke, the mean $t_{1/2}$ values were very similar with both data sets (25.6 hr in ng/ml vs 25.4 ng/mg creatinine). The correlation coefficient, however, was moderate ($r=0.58$).

Comparison of elimination data obtained from urine cotinine levels presented as ng/ml with data normalized by mg creatinine yielded some important results. Strong correlations were observed in all cases where initial urine cotinine concentrations were above 50 ng. For instance, cotinine elimination in nicotine gum users ($r = 0.79$) and urine cotinine elimination in nicotine gum users were also strongly correlated ($r=0.99$). However, the correlations were less strong when initial cotinine concentrations were low, as in smokers and nonsmokers exposed to sidestream smoke. The correlations were $r=0.60$ and $r=0.57$, respectively. It is important to note that these are still moderate correlations.

Current work with controlled human exposures is investigating differences in nicotine absorption when nonsmokers are exposed to machine-generated sidestream smoke or to environmental tobacco smoke produced by smokers in the same chamber. Differences in human smoking patterns (such as time between puff-drawing and inhalation) might affect the amount of sidestream smoke presented to the nonsmoker.

III. FUTURE STUDIES

A. Animal Bioassay

Short-term exposures to labelled SS are being planned for the current year. These studies will be conducted to determine the site of deposition of various smoke constituents within the animal respiratory tract.

B. Human Exposure Studies

It is of great importance to determine the dose of SS received by the most heavily exposed person, namely the smoker, and the relative contribution of SS to the carcinogenic potential of whole smoke.

Exposure studies to estimate the uptake of SS by smokers will be conducted. Several methods of differentiating SS uptake from MS uptake will be tried. These include selective capture of SS by cone filters or of MS by valves placed behind the cigarette filter. Additionally, the deposition of nicotine on the hair of smokers and nearby nonsmokers will be measured to determine the amount of environmental nicotine within the breathing zones of smokers and exposed nonsmokers.

**HISTOPATHOLOGICAL EVALUATION OF TRACHEA AND LARYNX
FOR SYRIAN GOLDEN HAMSTERS EXPOSED TO MS OR SS**

Table II

<u>Lesion</u>	<u>I</u>	<u>II</u>	<u>GROUP III</u>	<u>IV</u>	<u>V</u>
Focal epithelial hyperplasia and squamous metaplasia	1/20	1/20	3/20	4/60	7/60
Epithelial hyperplasia with small papilloma	0/20	0/20	0/20	5/60	2/60
Epithelial dysplasia	0/20	0/20	0/20	0/60	1/60

Group I - Control animals
 Group II MS sham
 Group III SS sham
 Group IV MS exposed
 Group V SS exposed

TABLE I

PERCENTAGE OF ANIMALS IN EACH GROUP SURVIVING THE BIOASSAY (A)

MONTHS	SHAM TREATED (B)	MAINSTREAM EXPOSED	SIDESTREAM EXPOSED
3	98	100	91
6	88	97	85
9	80	93	84
12	41	78	70
15	23	55	33
18	4	17	8

**A EACH EXPOSURE GROUP BEGAN WITH 30 MALES AND 30 FEMALES
WHILE SHAM TREATED GROUPS HAD 20 ANIMALS FOR EACH
SHAM PROTOCOL**

**B SURVIVAL DATA FOR MS-SHAM TREATED AND SS-SHAM TREATED
HAVE BEEN POOLED.**

2023381396

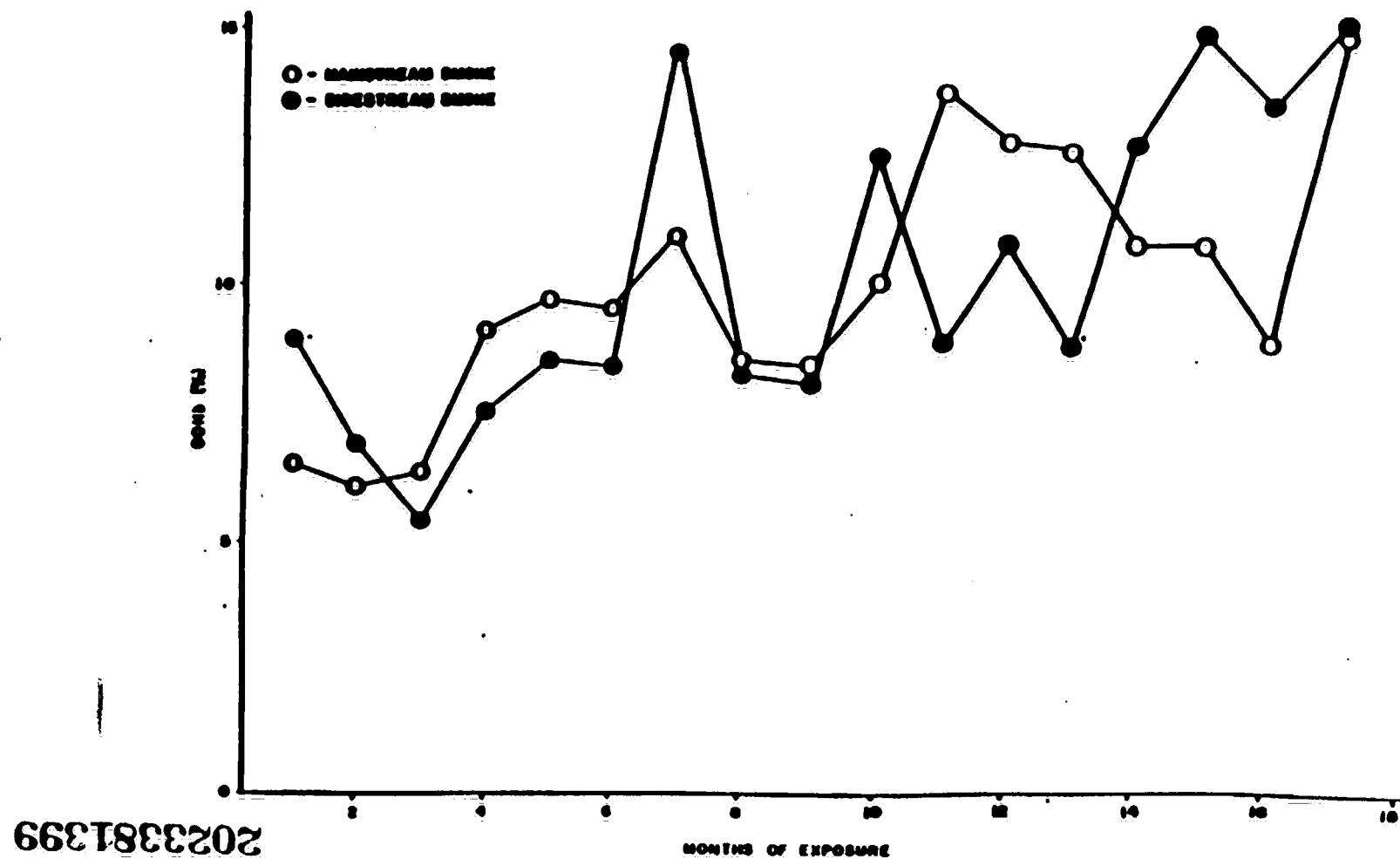
TABLE III

URINE COTININE HALF-LIFE TIME IN SMOKERS, USERS OF NICOTINE GUM AS WELL AS EX-SMOKERS AND NEVER-SMOKERS EXPOSED TO SIDESTREAM SMOKE

Nicotine Exposure	t 1/2 ng/cotinine ml	t 1/2 ng/cotinine mg/creatinine	Correlation Coefficient (r)	Intercept (ng cotinine/ mg creatinine)
Smokers				
A	- 15.3	- 12.1	- 0.80	2568
B	- 13.6	- 12.9	- 0.95	167
C	- 12.1	- 11.8	- 0.92	1388
D	- 18.7	- 15.1	- 0.89	3006
E	- 17.4	- 17.3	- 0.96	345
Nicotine Gum Users				
A	- 16.4	- 18.0	- 0.94	42
B	- 11.1	- 10.8	- 0.94	269
C	- 24.5	- 25.9	- 0.80	126
Sidestream Smoke/Ex-Smokers				
B	- 31.2	- 26.6	- 0.88	16
C	- 48.4	- 29.4	- 0.83	16
D	- 39.2	- 26.6	- 0.92	26
Sidestream Smoke/Never-Smokers				
	- 29.7	- 30.4	- 0.89	21
	- 23.3	- 28.5	- 0.95	62
	- 19.9	- 21.3	- 0.94	14
	- 33.4	- 25.4	- 0.88	20
	- 21.4	- 16.1	- 0.88	24

FIGURE 1

CARBOXYHEMOGLOBIN LEVELS IN MS AND SS EXPOSED SYRAN GOLDEN HAMSTERS



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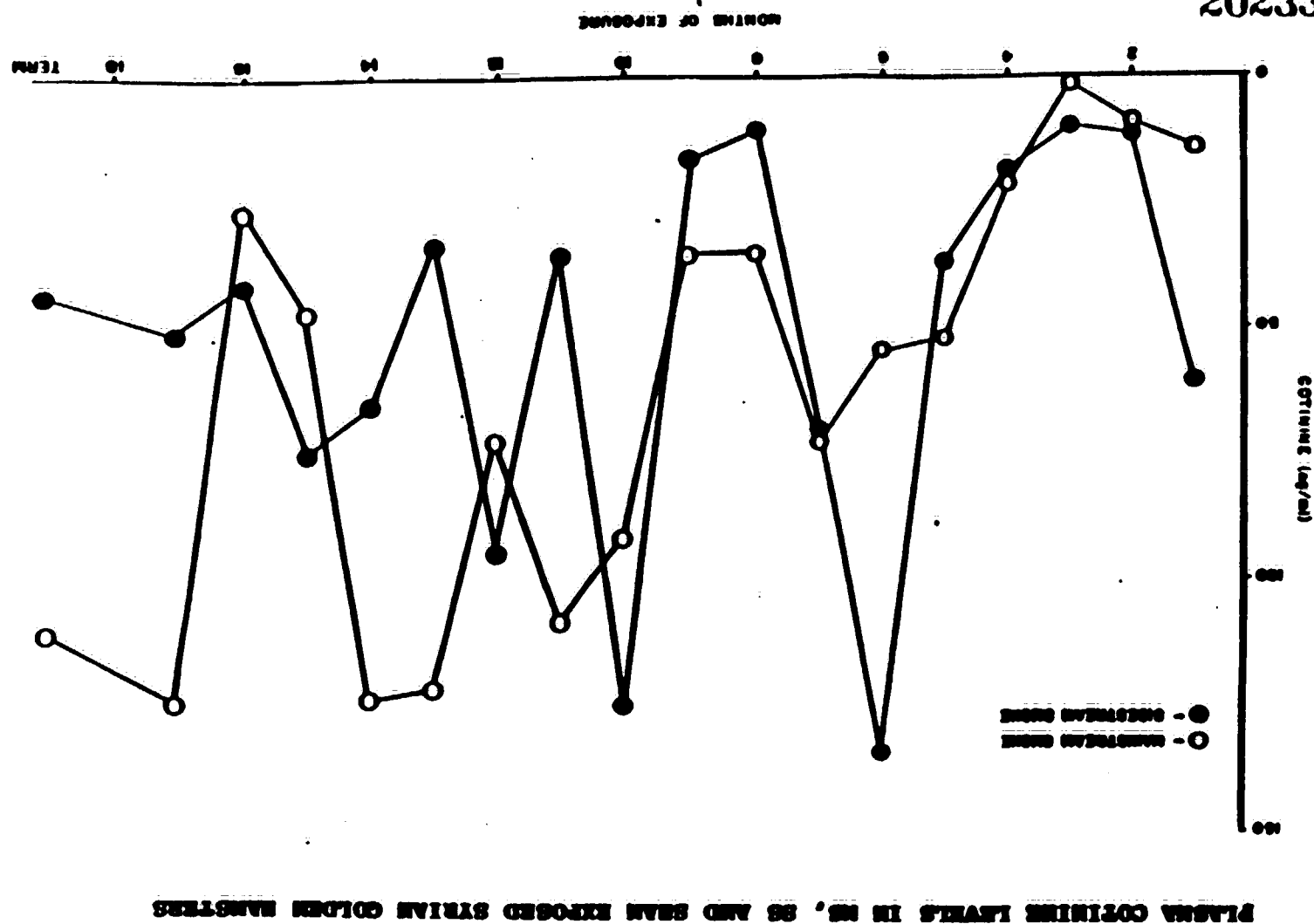
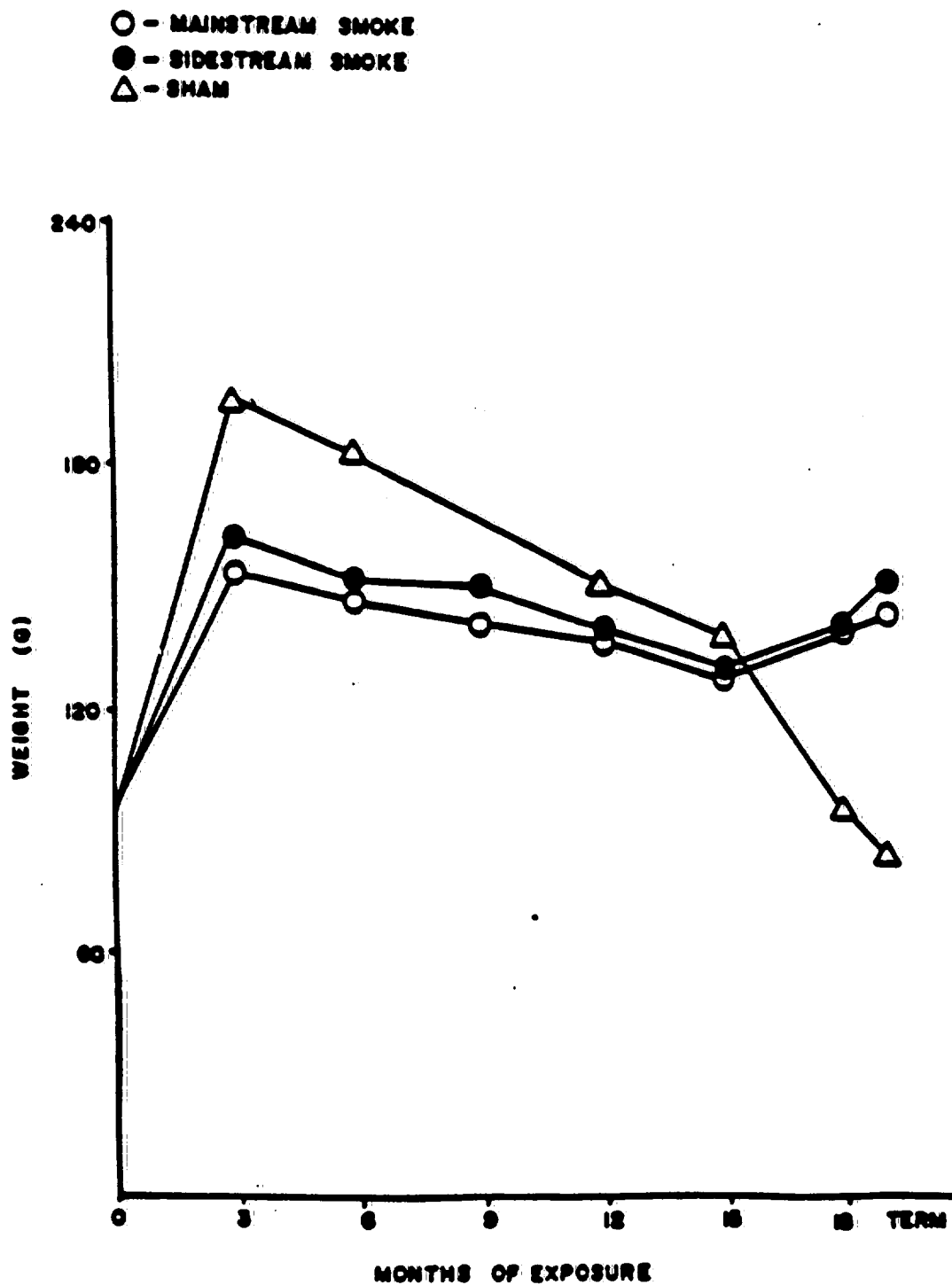


FIGURE 3

AVERAGE WEIGHTS OF EXPOSED AND SHAM EXPOSED ANIMALS DURING
18 MONTHS OF AN INHALATION BIOASSAY

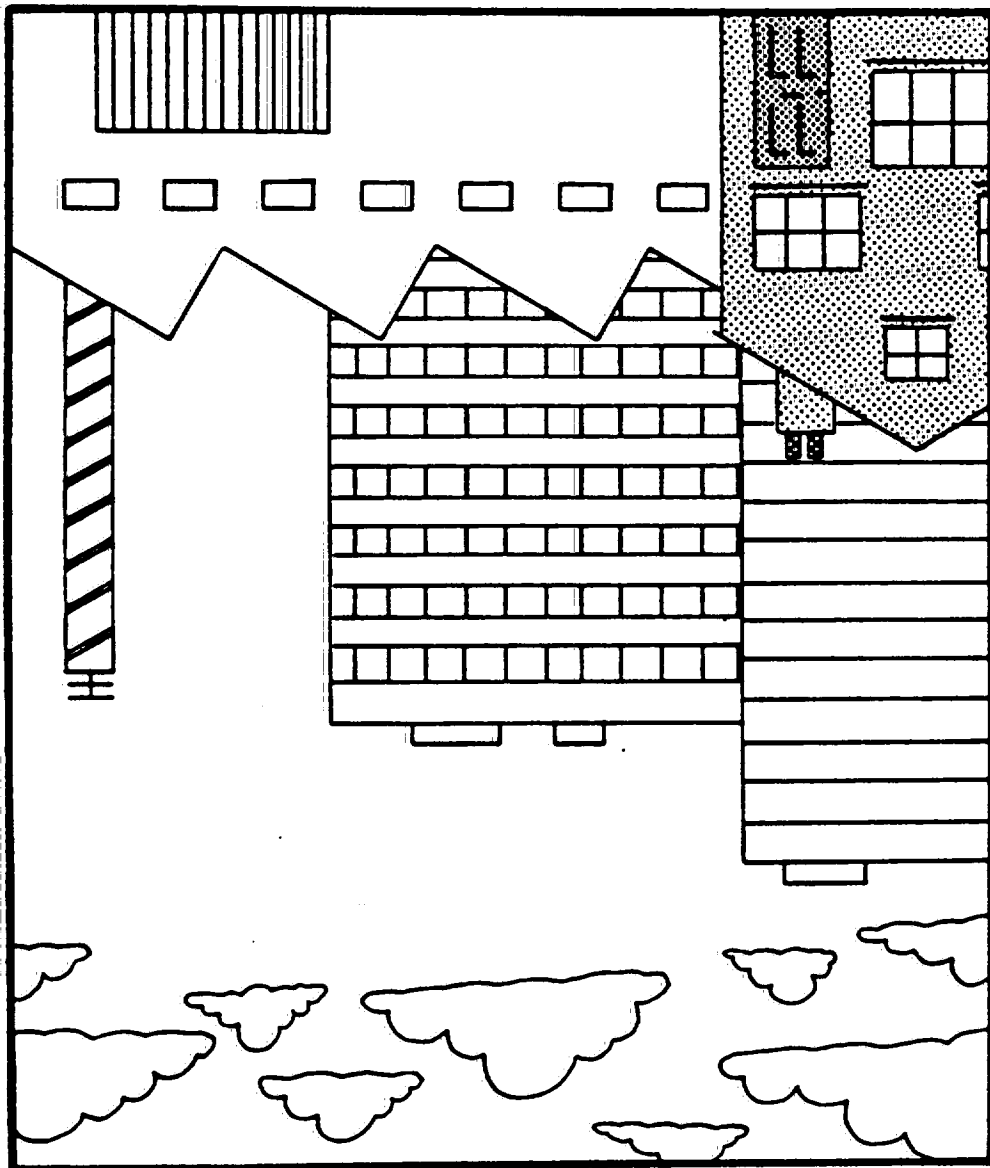


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Edited by R. Perry
and P.W. Kirk



INDOOR AND AMBIENT AIR QUALITY

EXPOSURE OF HAMSTERS AND RATS TO SIDESTREAM SMOKE OF CIGARETTES: PRELIMINARY RESULTS OF A 90-DAY-INHALATION STUDY

F. Adkater*, G. Scherer*, R. Wenzel-Hartung*, H. Brune* & C. Thomas*

Forschungsgesellschaft Rauchen und Gesundheit mbH, Friesenthal 2, D-2000 Hamburg 13, FRG

*Biologisches Laboratorium Dr med Hans Brune, c/o Vaseline-Werk, Würthdamm 13-17, D-2000 Hamburg 11, FRG

*Pathologisches Institut, Klinikum Lahnberge, Baldinger Straße, D-3650 Marburg/Lahn, FRG

ABSTRACT

Male and female F-344 rats and Syrian golden hamsters were whole-body exposed to sidestream smoke (SS) of Kentucky reference cigarettes type 2R1P. The exposure took place in a 35 m³ stainless steel chamber for 10 h per day, 5 days a week for up to 90 days. The 90 day exposure period was followed by a recovery period also of up to 90 days. The chamber was ventilated by 6 air changes per hour. Eight cigarettes were smoked simultaneously on an automatic smoking machine throughout the exposure period, resulting in a respirable mass concentration of about 4 mg/m³. The corresponding levels of carbonmonoxide (CO), nitrogen oxide (NO) and nitrogen dioxide (NO₂) were approximately 25 ppm, 600 ppb and 80 ppb, respectively. During the daily exposure regimen, carboxyhaemoglobin (COHb) increased by 2-3 % in both species. After the daily exposure, nicotine and cotinine serum concentrations in rats were found to be about 50-100 ng/ml and 200-400 ng/ml, respectively. The levels for hamsters were 30-50 ng/ml for nicotine and 80-120 ng/ml for cotinine. No exposure-related changes in the clinicochemical and haematological parameters were observed. Histopathological examination of the respiratory tract by light microscopy revealed no differences between sidestream smoke exposed animals, sham-exposed animals and untreated cage controls. Despite the absence of changes visible in the light microscope, it was decided to look at a small number of samples of trachea and lung parenchyma by electron microscopy. This investigation revealed suggestive evidence of slight changes in exposed animals which reversed for the most part or completely during the 90 days following the end of exposure.

INTRODUCTION

It has been alleged that exposure to ETS (environmental tobacco smoke) may increase the lung cancer risk in non-smokers (1). Mainly

three lines of evidence are cited in order to support this view: 1) ETS contains carcinogenic and mutagenic compounds as shown by chemical analysis and in vitro assays, 2) epidemiological studies show a relative risk between 1 and 2 for non-smoking wives of smokers when compared to non-smoking wives of non-smokers, 3) an excess risk has to be assumed when extrapolating from smoking to passive smoking (2). On the other hand, the presently available scientific evidence for a causal relationship between passive smoking and lung cancer is very weak, if it exists at all (3). Animal inhalation studies could provide valuable information on the carcinogenic potential of inhaled ETS or SS of cigarettes in the respiratory tract. Therefore we carried out a subchronic 90-day-inhalation study with rats and hamsters mainly addressing two questions: 1) What (if any) lesions are induced by exposure to high, but tolerable, concentrations of sidestream smoke in the respiratory tract during a period of up to 90 days? 2) Are any lesions that are produced reversible during a subsequent recovery period of up to 90 days? In order to mimic ETS exposure as closely as possible, a whole-body exposure regimen lasting for 10 h per day, 5 days per week was chosen.

METHODS

Experimental animals and exposure conditions

Fisher 344 rats (Charles River Wiga GmbH, Sulzfeld, West-Germany) and Syrian golden hamsters, strain Han: Aura (Zentralinstitut für Versuchstiersucht, Hannover, West-Germany) were randomly assigned to treatment and control groups as shown in Table 1.

During the daily exposure period (7 am to 5 pm) all animals were housed in steelwire cages (2 animals/cage) in a stainless steel chamber (35 m³) which was ventilated by 6 air changes per hour. In the chamber the rodent had access to drinking water but not to food. The air was drawn through the chamber by means of a pump installed at the outer downstream side of the chamber. An automatic smoking machine (RM 30/V, Borgwald, Hamburg, W.-Germany) was positioned in a hood at the outer upstream side of the chamber. The sidestream smoke of 8 simultaneously burning Kentucky reference cigarettes (2R1F, mainstream tar and nicotine: 23.4 mg and 1.74 mg, respectively) entered the chamber through an inlet (0.5 x 0.5 m) at the front wall. Eleven puffs were drawn per cigarette. The mainstream smoke was discarded. On the average, 448 cigarettes were smoked during the 10 h exposure period. The smoke was evenly distributed by means of 4 air jets which were installed at the inside edges of the inlet. Cage positions were routinely rotated on a daily basis. During the exposure-free time the animals were kept under usual laboratory conditions for rodents.

The sham-exposed groups were treated in exactly the same way, except that the smoking machine attached to the sham-chamber ran without cigarettes.

Table 1: Experimental groups and number of animals

Group	Treatment	R a t s		H a m s t e r s	
		Male	Female	Male	Female
1	Exposure with SS (10 h/d, 5 d/week for 90 days)*	65	35	65	35
2	Recovery (as group 1, after exposure period 90 days under room control conditions)**	40	-	40	-
3	Sham control (as group 1 and 2 exposure to fresh air instead of SS)	80	35	80	35
4	Room control (no treatment)	55	40	55	40

* Subgroups of 5 to 20 animals were killed at the following time points: 0, 1, 3, 5, 12, 26, 47, and 90 days after start of exposure. Female animals were sacrificed 0, 47 or 90 days after start of the experiment.

** Subgroups of 5 to 20 animals were killed at the following time points: 120, 150, and 180 days after start of exposure.

Chamber monitoring

During the exposure period, CO (UNOR 6 W, Mairak, Hamburg, FRG), NO/NO₂ (ML 8840, Monitor Technologies GmbH, Allershausen, W.-Germany) and particulate mass (RAM 1, Klaus Schiffer GmbH, Langen, W.-Germany) were recorded continuously. The particle measurements were calibrated by a gravimetric method (4). Nicotine (5), carbonyls (6, 7) ammonia (8), benzo(a)pyrene (9) and N-nitrosodimethylamine (10) were measured at regular intervals according to modified published methods.

Biological parameters

At the time points of interim killing COHb (IL 282, Instrumentation Laboratories, USA) as well as nicotine (11) and cotinine (12) in serum were measured as dosimetric parameters. The usual clinicochemical and haematological examinations were carried out on blood taken from animals at the time of sacrifice. Body weight was measured every week. Food consumption during four consecutive days was determined on four occasions.

Histopathology

All organs were examined macroscopically at necropsy and found to be without pathological changes.

A detailed post mortem examination was carried out on all animals and changes in any organ or tissue were recorded. The following organs were removed and fixed in 10 % formalin: Nasal cavity, larynx, trachea, lung (distended with air), liver, kidney, adrenals, brain, pituitary, urinary bladder, heart, tongue, thyroid, parathyroid, thymus, mammary gland, testes or ovaries. The organs were sectioned, stained (haematoxylin-eosin and/or PAS), and the slides were examined by light microscopy.

Electron microscopy

One sample of lung and one of trachea from 4 exposed and 4 sham-exposed male rats and hamsters killed at 90 days and from 2 exposed male rats and 4 exposed male hamsters killed 90 days after the end of exposure were fixed in glutaraldehyde for e.m. examination.

RESULTS

The results of the chamber monitoring are summarized in Table 2. All substances measured were fairly evenly distributed in the experimental room. Size distribution measurements with a 10-level-impactor revealed a maximum of particle mass at 0.3 μ m. No particles could be measured beyond 2 μ m.

Table 2: Average air concentrations in the exposure- and sham-chambers

	Exposure	Sham
Particles (mg/m^3)	4.3	<0.1
CO (ppm)	25	1
NO (ppb)	400	60
NO ₂ (ppb)	70	nd*
Nicotine ($\mu\text{g}/\text{m}^3$)	1000	2
Formaldehyde ($\mu\text{g}/\text{m}^3$)	600	6
Acetaldehyde ($\mu\text{g}/\text{m}^3$)	1200	4
Acrolein ($\mu\text{g}/\text{m}^3$)	450	11
Ammonia (mg/m^3)	3.1	0.1
Dimethylnitrosamine (ng/m^3)	200	25
Benzo(a)pyrene (ng/m^3)	56	nd*

* nd = not detectable.

With the exception of a sham-exposed hamster who was found dead after 10 weeks, all the animals in all the groups remained in good health without obvious differences between the groups.

COHb, nicotine and cotinine serum levels after a daily exposure period are shown in Table 3. COHb increased about 3 % in both species. Nicotine and cotinine serum concentrations were substantially higher in exposed rats than exposed hamsters.

Table 3: Results of dosimetric measurements in smoke-exposed and sham-exposed male rats and hamsters at the end of the 90-day-exposure period:

	Mean (SD) (n=16)			
	Smoke-exposed		Sham-exposed	
	Rats	Hamsters	Rats	Hamsters
COHb (%)	3.3 (0.6)	3.1 (0.4)	0.8 (0.5)	0.3 (0.1)
Serum nicotine (ng/ml)	99 (40)	51 (20)	3 (2)	3 (3)
Serum cotinine (ng/ml)	350 (68)	115 (40)	3 (3)	0.5 (1)

The body weight gain of exposed, sham-exposed and control male and female rats were similar throughout the 13 week exposure period. However, following the termination of smoke exposure, the male reversibility rats gained more weight than the sham-exposed and untreated controls. In contrast to the untreated male hamsters, the exposed and sham-exposed male hamsters gained no weight during the exposure period. During the first 90 days of the study the exposed and sham-exposed female hamsters exhibited a small increase in weight, but this was much less than the gain in weight of the untreated female hamsters. After the termination of exposure, the reversibility and sham-exposed hamsters gained more weight than the untreated controls. After 180 days neither the male 90-day exposed rats and hamsters nor the sham-exposed animals showed any significant difference in body weight as distinct from the untreated animals.

The daily food intake of the male and female exposed, sham-exposed and untreated rats was very similar. Compared with the sham-exposed and untreated hamsters the food intake was less in male and female exposed hamsters during the exposure period.

No differences between treated and control groups were found in the clinicochemical and haematological parameters.

Histopathological investigation by light microscopy revealed no exposure-related changes in the lower respiratory tract (lung and trachea) or in any of the other organs examined. Preliminary electron microscopy of the lung and trachea revealed the following changes in the smoke-exposed animals after 90 days: lungs, increased numbers and hypertrophy of type II pneumocytes, increased numbers of alveolar macrophages and thickening of the basal membrane; trachea, reduction in number of ciliated cells. These changes could be observed in both species. They were, however, more marked in rats than in hamsters. Ninety days after termination of

the exposure these lesions were found to have completely or for the most part disappeared.

DISCUSSION

One of the aims of this pilot subchronic inhalation experiment, was to detect early lesions (if any) which could be induced by an unrealistically high, but tolerable sidestream smoke exposure dose in rats and hamsters. The exposure level chosen was a particle mass concentration of about 4 mg/m^3 which parallels several recent long-term diesel exhaust inhalation studies (13). This concentration is up to 100 times higher than the smoking-related particle levels reported for real-life situations (1). The nicotine uptake by the smoke-exposed animals, as indicated by serum nicotine and cotinine concentrations, is also between one and two orders of magnitude higher than that found in humans passively exposed to tobacco smoke (2). The exposure conditions were well tolerated by the rodents. The small reduction in weight gain observed in hamsters is clearly attributable to the stress by being confined in exposure chambers as can be deduced from the similar effect in the sham-exposed hamsters.

No treatment-related changes were found in the respiratory tract by light microscopy. Limited evidence derived from e.m. studies on a small number of samples suggested that exposure damaged ciliated cells in the trachea and lung parenchymal cells, but these effects disappeared during 90 days following the end of exposure. The most obvious of these changes were an increase in the number of alveolar macrophages, the occurrence of pigmented macrophages in the lung parenchyma and partial deciliation of tracheal epithelial cells. Rats exposed to diesel exhaust of a concentration of 6.3 mg/m^3 , 8 h/d for 5 days or longer are reported not to have cleared the diesel particulate from the lungs 90 days after the end of the exposure (14). It is assumed that the particle load of the lungs is the main cause of the induction of lung tumors in diesel exhaust-exposed rats (13).

The outcome of this subchronic inhalation study suggests that the particle burden of the lung of rodents exposed to sidestream smoke is much less than that in diesel-exposed animals. From our results we further conclude that the application of this exposure regime in a long-term study with hamsters and rats is feasible. On the other hand, an exposure dose of 4 mg/m^3 might lead to some alterations of the mucociliary escalator. It is extremely unlikely that these lesions are caused by passive smoking in humans under real-life situations since they are rarely observed in non-smokers (15). These observations possibly differing between animal and man have to be considered before any conclusions are drawn.

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4. R.D. Treitman, J.D. Spengler and T. Tosteson, Exposure

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4. R.D. Treitman, J.D. Spengler and T. Tosteson, EXPOSURE

SUSPECTED PULMONARY CARCINOGENS IN ENVIRONMENTAL TOBACCO SMOKE

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(Received 12 May 1988, accepted 25 May 1988)

ABSTRACT

Among 50 environmental tobacco smoke constituents listed as potentially harmful to human health, 17 are designated as suspect carcinogens by either the U.S. National Toxicology Program, or the International Agency for Research on Cancer, or both. The 17 constituents have not been adequately shown to cause pulmonary cancer via inhalation in animals. On the other hand, pulmonary carcinogens not present in environmental tobacco smoke, such as bis-chloromethyl ether, are supported by adequate human and animal inhalation studies.

INTRODUCTION

Recent reports from the U.S. Surgeon General (1), the U.S. National Academy of Science - National Research Council (2, 3), and the International Agency for Research on Cancer (IARC) (4), have made a "consensus" list of potentially harmful constituents of environmental tobacco smoke (ETS). Among the selected fifty constituents, 17 are designated suspect carcinogens by either the U.S. National Toxicology Program (USNTP) (5), or the International Agency for Research on Cancer (4), or both. The purpose of this presentation is to discuss the above mentioned reports in terms of pulmonary carcinogenesis. In December 1987, the most comprehensive database became available to scientists interested in environmental and occupational health. The Registry of Toxic Effects of Chemical Substances (RTECS), hitherto available only to personnel of National Institute of Occupational Safety and Health, can be accessed through the National Library of Medicine (Medline) (6). The unique feature contained in the RTECS database for over 90,000 substances is a uniform expert evaluation of animal testing results for carcinogenesis. A uniform standard has been adapted to differentiate between "equivocal" and "unequivocal" results of suspected carcinogens administered by various routes. The following discussion is intended as a "review of reviews" on ETS constituents suspected by some of causing pulmonary cancer.

REFERENCE STANDARD FOR INHALED PULMONARY CARCINOGENS

Prior to the discussion of ETS constituents, it is necessary to briefly describe a reference pulmonary carcinogen such as bis-chloromethyl ether. Exposure of workers to this substance increases the risk of lung cancer, mainly, oat-cell carcinoma. The exposure occurs in chemical plant workers, ion exchange resin makers, laboratory workers, and polymer workers. Absorption of the chemical is through the lungs and skin. It is rated by USNTP as a "known carcinogen" because there is "sufficient evidence of carcinogenicity from studies in humans", which suggests a causal relationship between the agent and lung cancer (5).

Bis-chloromethyl ether produced tumors at the site of application in mice and rats, i.e., in the lungs after inhalation, subcutaneous tissues after

injection, and in the skin after repeated topical application. The features of inhalation studies are as follows (6):

- rat inhalation - 100 ppb for 6 hours daily for 6 weeks;
- rat inhalation - 100 ppb for 6 hours daily for 26 weeks;
- rat inhalation - 75 ppb for 6 hours daily for two years;
- mouse inhalation - 1 ppm daily for 82 days.

The above results support the rating by IARC of "sufficient evidence" as an animal carcinogen (5) and the rating by USNTP of "known carcinogen." The threshold limit value (TLV) is 0.001 ppm, which provides an acceptable factor of safety based on additional short-term inhalation studies in humans and animals (6).

REFERENCE STANDARD FOR ENVIRONMENTAL TOBACCO SMOKE CONSTITUENTS

Benzo[a]pyrene is the most widely investigated constituent of tobacco smoke and has been detected in mainstream smoke, sidestream smoke and ETS (1-4). In the accepted terminology of USNTP, this constituent is not a "known carcinogen" but "may reasonably be anticipated to be carcinogen" (5). The primary reason for this rating is a lack of human epidemiologic data showing that exposure to benzo[a]pyrene alone is a risk factor for cancer in humans. The lack of human epidemiologic data relates to the IARC rating of "sufficient evidence" for animal cancer, but not for human cancer.

Tumors have been reported at the site of administration of benzo[a]pyrene, such as dermal, subcutaneous, intramuscular, intracerebral, intraperitoneal, and rectal areas. The results of intrathoracic routes are as follows (6):

- dog intrathoracic implant - 651 mg/kg continuously for 2 weeks;
- rat intrathoracic implant - 150 ug/kg continuously for 2 weeks;
- mouse intratracheal injection - 200 mg/kg intermittently for 10 weeks;
- rat intratracheal injection - 200 mg/kg intermittently for 15 weeks;
- rabbit intratracheal injection - 145 mg/kg intermittently for 2 years;
- hamster intratracheal injection - 120 mg/kg intermittently for 17 weeks;
- hamster intratracheal injection - 360 mg/kg intermittently for 36 weeks;
- hamster inhalation - 9500 ug/cuM 4 hours intermittently for 96 weeks;
- mouse inhalation - 200 ng/cuM 6 hours intermittently for 13 weeks.

In the above listed testings, RTECS has rated inhalation and most other results as an "equivocal tumorigenic agent" yielding "uncertain but seemingly positive" results (6). The positive results not rated as "equivocal" were derived by intrathoracic implantation or intratracheal injection. Only the inhalation study is relevant to the health effects of ETS, but the results are designated "equivocal".

The concentrations of benzo[a]pyrene were reported as 9500 ug/cuM in the hamster, and 200 ng/cuM in the mouse. The peak benzo[a]pyrene concentration reported in the literature is 0.14 ug/cig or 140 ng/cig for sidestream smoke emitted by one cigarette. The mixture inhaled in one hamster study consisted of 9500 ug, which, when divided by 0.14 ug, is equivalent to 67857 burning cigarettes contained in one cuM. In the mouse study, the mixture consisted of 200 ng (divided by 140 ng equals 1.43 burning cigarettes in one cuM of inspired air), and the results were "equivocal". Although the concentration in the mouse is lower than that used in the hamster (by a factor of 47452), even the "equivocal" results are still inapplicable to ETS. The estimate of 67857 burning cigarettes per cuM of enclosure would be intolerable in terms of mucosal irritation; so that a person would not remain in the enclosure long enough to simulate conditions of animal inhalation experiments.

Benzo[a]pyrene is one of 46 polycyclic aromatic hydrocarbons (PAH) contained in mainstream smoke, but only ten have been detected in ETS (4). The following five PAH's have been tested by skin painting only: benzo[a]fluorene,

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39 ng/cuM; benzo[gi]perylene, 5.9 to 17 ng/cuM; coronene, 0.3 to 2.8 ng/cuM; dibenz[a,j]anthracene, 6 ng/cuM; and perylene, 0.1 to 11 ng/cuM. The above corresponding ranges of concentration in ETS are considerably less than that for benzo[a]pyrene (2.8 to 760 ng/cuM).

Benzo[a]pyrene is a research chemical and only researchers would be exposed to the pure substance. However, occupational exposure is widespread because benzo[a]pyrene is present in coal tar, coke oven emissions, and creosote, that have corresponding work exposure standards (5). Benzo[a]pyrene and other PAH's occur in the combustion products of coal, oil, petroleum and biologic matter. Human exposure can occur indoors as a result of heating and cooking with natural gas, oil, coal, and wood. Individuals working at restaurants, airports, tarring facilities, refuse incinerators, power plants and coke manufacturing facilities may be exposed to benzo[a]pyrene and related polynuclear aromatic hydrocarbons.

"SUSPECTED CARCINOGENS" IN ENVIRONMENTAL TOBACCO SMOKE

Among the "consensus" selection of fifty health hazardous substances (1-4), there are 17 "suspected carcinogens" by either the USNTP (5), or IARC (4), or both. The following table lists 17 substances in decreasing order of amounts in sidestream smoke (SSS, expressed as mg/cigarette). It should be noted that the total sidestream smoke emissions are derived from maximal values reported in the literature (1-4). The column on Chemical Abstract Service (CAS) registry numbers did not appear in cited reviews and are added in Table 1 to establish accuracy in identifying biomedical literature for 17 "suspected carcinogens".

Table 1. Sidestream Constituents and Primary Sources.

NTP Line	CAS No.	SSS Constituents	SSS:mg/cig	Primary Sources
	630-08-0	Carbon monoxide	108	BC I
	54-11-5	Nicotine	8.2	T
(a)	50-00-0	Formaldehyde	5.0	HIP
(b)	71-43-2	Benzene	0.48	HIP
(c)*	16543-55-8	Nitrosornicotine	0.009	T
(d)*	62-75-9	Nitrosodimethylamine	0.004	I R
(e)	?	NNK**	0.004	T
(f)	95-53-4	2-Toluidine	0.003	I
(g)	7440-02-0	Nickel	0.0024	IP
(h)	55-18-5	Nitrosodiethylamine	0.001	IPR
(i)	930-55-2	Nitrosopyrrolidine	0.0009	B W
(j)	7440-43-9	Cadmium	0.00072	IP
(k)	56-55-3	Benz[a]anthracene	0.00028	C R
(l)	92-67-1	4-Aminobiphenyl	0.00014	I R
(m)	50-32-8	Benzo[a]pyrene	0.00014	C R
(n)	301-01-2	Hydrazine	0.00009	IP
(o)	116-54-7	Nitrosodiethanolamine	0.00008	P W
(p)	91-59-8	2-Naphthylamine	0.00005	I R
(q)*	7440-68-1	Polonium 210	(pCi 0.4)***	HI

* Not in USNTP Annual Report on Carcinogens (5); Suspected carcinogen in IARC publications(4)

** 3-Pyridyl-3,3(N-methyl-N-nitrosoamino)propylketone

*** picocurie

The above table includes nicotine and carbon monoxide for the purpose of comparison. Nicotine is often designated the most pharmacologically active constituent of tobacco smoke (8,9), but is not a suspected carcinogen. It is present in ETS at concentrations ranging from 3 to 38 ug/cuM (10). Carbon monoxide also is not a suspected carcinogen and is present in ETS in concentrations ranging from 0.5 to 35 mg/cuM (11). The presence of carbon monoxide in ambient air represents contributions from tobacco smoke as well as from combustion of fuels and from biologic processes. Nicotine, nitrosonicotine and NNK are the only constituents in the above table that originate solely from tobacco smoke. The 17 tabulated constituents (a to q) originate primarily from sources other than tobacco (T), namely, Biologic matter; Combustion; Household pollution; Industrial chemical manufacturing; Products personal and public use; Research use; and Waste, both solid and liquid.

SIDESTREAM SMOKE AND ENVIRONMENTAL TOBACCO SMOKE

The total sidestream emissions for carbon monoxide, nicotine and 17 "suspected carcinogens" are expressed as mg per cigarette. The highest amount (108 mg) is for carbon monoxide, and the lowest amount (0.00005 milligram or .05 microgram, or 50 nanograms) is for (p) 2-naphthylamine. The microgram (ug) and nanogram (ng) quantities of sidestream emissions suggest that hundreds or even thousands of cigarettes would need to be ignited in an enclosure in order to reach hazardous levels in the environment.

Threshold Limit Values (TLV)

In the workplace, threshold limit values are available for some substances under consideration (7):

Table 2. Cigarette Sidestream Emissions to Attain TLV Levels.

Line	SSS Constituent	SSS:mg/cig	TLV mg/cuM	Cig. Equivalence per 100 cuM
(a)	Formaldehyde	5.0	1.5	30
(g)	Nickel	0.0024	0.1	4166
(b)	Benzene	0.48	30	6250
(j)	Cadmium	0.00072	0.05	6944
(n)	Hydrazine	0.00009	0.1	11111
(f)	2-Toluidine	0.003	9	300000
(q)	Polonium 210	(see text)		2500000
(m)	Benzo[a]pyrene	(see text)		6785700

The estimate for cigarette equivalence is for sealed non-ventilated enclosure of 100 cubic meters. Based on total sidestream emission per cigarette and TLV, it would take 300000 burning cigarettes to approach the respective TLV for 2-toluidine (9 times 100 divided by 0.003). The cigarette equivalence for Polonium 210 is estimated as 2500000 based on "action level" for radon indoors (12). As stated above the benzo[a]pyrene hazardous level is derived from transposition of concentrations used in hamster inhalation study.

Experimental Pulmonary Carcinogenesis

The suspicion that each of 17 ETS constituents causes cancer is based on

results of animal testing such as the following (6):

skin painting resulting in skin tumors: (d) nitrosodimethylamine; (h) nitrosodiethylamine; (k) benz[a]anthracene and others.

added to drinking water causing liver and urinary bladder neoplasms:

(d) nitrosodimethylamine; (e) NKK; and (o) nitrosodiethanolamine.

added to diet causing liver and bladder neoplasms: (f) toluidine; (l) aminobiphenyl; and (s) naphthylamine.

oral gavage or intraperitoneal injection causing pulmonary and extrapulmonary neoplasms: (c) nitrosonornicotine; (e) NNK; (h) nitrodiethylamine; (i) nitrosopyrrolidine; (n) hydrazine; and (k) benz[a]anthracene.

intratracheal injection causing pulmonary neoplasm: (m) benzo[a]pyrene.

inhalation causing pulmonary neoplasm: (m) benzo[a]pyrene.

inhalation causing intraabdominal neoplasm: (d) nitrosodiethylamine.

inhalation causing leukemia: (b) benzene.

inhalation causing nasal tumor: (a) formaldehyde.

inhalation causing tracheal neoplasm: (g) nickel; (j) cadmium.

It should be noted that nearly all constituents listed as carcinogens in the study were not tested by inhalation routes, but instead, by oral, intraperitoneal, dermal and intratracheal administration. The last mentioned route should not be confused with inhalation for several reasons relating to absorption, disposition and metabolism (Table 3). The five substances in the list tested by inhalation are: (a) formaldehyde, (b) benzene, (g) nickel, (j) cadmium, and (m) benzo[a]pyrene. All five have been identified in miniscule quantities in sidestream smoke and originate primarily from processes and substances unrelated to tobacco smoke, i.e., building materials and auto emissions. As stated above, the results of benzo[a]pyrene inhalation testing are "equivocal".

Table 3. Comparative Physiology Resulting from Inhalation Versus Intratracheal Injection.

<u>Pulmonary Function</u>	<u>Inhalation</u>	<u>Intratracheal Injection</u>
Anesthesia	no	necessary
Vehicle	air	excipient or solvent
Oropharyngeal trauma	no	yes
Administered dose	simulate human	impractical
Protective cough reflex	present	subdued
Mucociliary transport	efficient	overload
Absorption	limited by exposure	prolonged beyond injection
Extrapulmonary metabolism	limited by exposure	continuously

Experimental carcinogenesis relating to ETS inhalation can only be investigated by exposing animals in an appropriate chamber (13). Inhalation exposures of animals have assisted in identifying carcinogens in the workplace, such as bis-chloromethyl ether (see above). Since the relevant exposure route for ETS constituents is limited to the respiratory tract, extrapulmonary routes are not germane. The intratracheal route cannot be used to prove or disprove pulmonary carcinogenicity because of alterations in lung function relating to absorption, elimination and pharmacokinetics. Intratracheal injection has been a useful tool for research on enzymatic, immunologic and histochemical changes that relate to carcinogenesis. However, the procedure has not been used for bioassay testing which is exclusively limited to the inhalation route for substances absorbed through the respiratory tract.

CONCLUSIONS

The criteria for proving that ETS contains pulmonary carcinogens have not been met. The available information for 17 alledged carcinogens detectable in ETS is limited to animal experiments, mostly consisting of dermal, oral, and parenteral injections. Intratracheal injection influences physiologic and biochemical events in the lungs to the extent that results cannot be applied to ETS exposure. Furthermore, for ETS constituents with workplace standards, hundreds or even thousands of cigarettes need to be ignited continuously for ETS to reach designated "carcinogenic" levels in an enclosure. The absence of relevant human or animal inhalation studies does not support the hypothesis that the 17 constituents in ETS are pulmonary carcinogens. The possibility of potentiation or antagonism among the constituents has not been proved or disproved, nor do the data exclude extrapulmonary carcinogenicity following oral administration or workplace exposure to levels considerably higher than those of ETS.

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Health Effects of 50 Selected Constituents of Environmental Tobacco Smoke

D. M. Aviado

Summary

Recent monographs from IARC, NAS-NRC, and USPHS Surgeon General include a common list of 27 particulates and 23 vapors allegedly responsible for health effects of environmental tobacco smoke (ETS). More than half of listed constituents have workplace standards. It takes from less than one to eight cigarettes for total sidestream emission (in an enclosed unventilated space of 10 m^3) to exceed threshold limit values (TLV) for nicotine, acrolein, formaldehyde, carbon monoxide, and ammonia. For each of 21 constituents, 50 to 29,600 cigarettes would have to be consumed in 10 m^3 to exceed respective TLVs. In all, 11 ETS constituents are suspected tumorigens based entirely on oral, dermal, subcutaneous, and/or tracheal injection in experimental animals. There are six ETS constituents that are in vitro mutagens and seven constituents with no known mutagenicity. The sidestream emission for each constituent is so low that any health consequence is inconceivable. In summary, there are no published animal experiments or human studies indicating that repeated exposure to any one of 50 ETS selected constituents can cause pulmonary tumors.

Introduction

During the past year, biomedical literature on environmental tobacco smoke has been reviewed by three agencies, namely, the Office of the United States Surgeon General, USPHS [1], the National Academy of Sciences - National Research Council, NAS-NRC [2], and the International Agency for Research on Cancer, IARC [3]. For the first time, the three supposedly independent groups have agreed on a selection of fifty biologically active constituents in environmental tobacco smoke (ETS). The selection was derived from over 150 constituents of sidestream smoke (SSS) analyzed chemically by Elliott and Rowe [4], Hoffmann et al. [5], Klus and Kuhn [6], Schmeltz et al. [7] and Sakuma et al. [8-10]. In reality, only 14 of the 50 selected constituents of cigarette smoke have been detected in environmental tobacco smoke. The other constituents have not been examined because of technical difficulties in analyzing microgram (10 to minus 6) and nanogram (10 to minus 9) concentrations. The 50 constituents comprise 1.3% of over 38,000 chemical substances identified in mainstream smoke (MSS). According to Duke and Green [11], the detection of 3,800+ constituents of cigarette smoke is largely due to recent refinements in collection, separation and analysis introduced by tobacco chemists. Whereas in 1964, there were only 500+ known smoke constituents and 16+ biologically active constituents [12], in 1987 there are 3,800+ MSS constituents, including 50 that are biologically active. This presentation focuses on these 50 constituents highlighted in recent monographs released by USPHS, NAS-NRC and IARC.

H. Kasuga (Ed.) Indoor Air Quality
© Springer-Verlag, Berlin Heidelberg 1990

2023381416

Table 1. Carbon monoxide (CAS 630-08-0)

Environmental Tobacco Smoke (ETS)	
Experimental chambers and semi-ventilated rooms	45.0-38.0 ppm
Offices and conference rooms	32.5- 2.5 ppm
Restaurants, bars, taverns and night clubs	30.0- 0.5 ppm
Work places	29.4- 2.8 ppm
Moving vehicles	30.0- 2.0 ppm
Threshold Limit Value (TLV)	
	50 ppm
	55 mg/cubic m
	550 mg/10 cubic m
Main-Stream Smoke (MSS)	23-10 mg
Side-Stream Smoke (SSS)	108-25 mg
Ratio SSS/MSS	4.7- 2.5
Calculation of maximal cigarette equivalent:	
550 mg divided by 108 = 5 cigarettes in 10 cubic meters	

Threshold Limit Value

The most widely used estimate of air quality in the workplace is the Threshold Limit Value (TLV). The TLV is determined by toxicologists, epidemiologists, and hygienists for the American Conference of Governmental Industrial Hygienists [13]. The recommended concentration of a substance, expressed in mg/cubic meter, or in parts per million (ppm), is the maximal level that should not be exceeded to prevent occupational disease. The TLV is arrived at by interpretation of the literature relating to human exposure level, human accidental deaths, if any, and animal lethality; lowest toxic concentration and highest nontoxic concentration derived from case reports and animal experiments; absorption, excretion and kinetics; toxic effects on skin, mucosa, muscles, nervous system, liver, kidneys, blood, reproductive organs, heart and lungs; and experimental induction of neoplasm.

Carbon monoxide is the most widely investigated constituent of environmental tobacco smoke (Table 1). The reported concentrations in public places rarely exceed the TLV of 50 ppm [14]. An essential step in the following discussion of biologically active constituents of cigarette smoke is calculation of "cigarette equivalent" defined as the number of cigarettes generating sidestream smoke (SSS) collected in a sealed enclosure of 10 cubic meters. For carbon monoxide, the maximal amount of SSS is 108 mg which is more than 4 times higher than MSS, representing the SSS/MSS ratio of 4.7. The TLV is defined as the safe level not to be exceeded to prevent occupational disease. For carbon monoxide, the TLV of 50 ppm is equivalent to 55 mg/cubic meter, or 550 mg/10 cubic meters. The TLV of 550 mg is divided by 108 mg SSS, which equals 5 cigarettes consumed in 10 cubic meters. In other words, it takes ignition of 5 cigarettes to maintain the TLV for carbon monoxide in a sealed enclosure of 10 cubic meters.

Milligram, Microgram, and Nanogram Quantities

The fifty selected cigarette smoke constituents that are biologically active (1-3) are listed in Table 2. Half of the chemicals are noted as "V" in the first column to mean vapors and gases, such as line 1V (carbon dioxide) and Line 2V (carbon monoxide); the remaining

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Table 2. Fifty selected ETS constituents

Line	CAS	Chemical name	SSS mg	MSS mg	SSS/MSS
1V	124-38-9	Carbon dioxide	440-16	40-20	11.0-8.0
2V	630-08-0	Carbon monoxide	108-25	23-10	4.7-2.5
3P		Particulates	76-20	40-15	1.9-1.3
4V	7664-41-7	Ammonia	22-10	1.3-0.5	170-40
5P	54-11-5	Nicotine	8.2-2.6	2.5-1.0	3.3-2.6
6V	108-88-3	Toluene	8.0-0.6	0.2-0.1	8.3-5.6
7V	10024-97-2	Nitrogen oxide	6.0-0.4	0.6-0.1	10.0-4.0
8V	50-00-0	Formaldehyde	5.0-0.7	0.10-0.07	50.0-0.1
9V	64-19-7	Acetic acid	2.9-0.6	0.81-0.33	3.6-1.9
10V	74-87-3	Methyl chloride	1.98-0.25	0.60-0.15	3.3-1.7
11V	107-02-8	Acrolein	1.50-0.48	0.10-0.06	15.0-8.0
12V	67-64-1	Acetone	1.25-0.20	0.25-0.10	5.0-2.0
13V	1121-55-7	3-Vinylpyridine	1.20-0.22	0.03-0.01	40.0-20
14V	110-86-1	Pyridine	0.80-0.10	0.04-0.02	20.0-6.5
15V	64-18-6	Formic acid	0.78-0.29	0.49-0.21	1.6-1.4
16V	71-43-2	Benzene	0.48-0.12	0.05-0.01	10.0
17V	108-99-6	3-Methylpyridine	0.47-0.04	0.04-0.01	13.0-3.0
18P	108-95-2	Phenol	0.42-0.08	0.14-0.06	3.0-1.6
19V	154-23-4	Catechol	0.32-0.06	0.36-0.10	0.9-0.6
20P	123-31-9	Hydroquinone	0.27-0.08	0.30-0.11	0.9-0.7
21V	74-89-5	Methylamine	0.18-0.05	0.03-0.01	6.4-4.2
22V	74-90-8	Hydrogen cyanide	0.12-0.04	0.50-0.40	0.2-0.1
23P	50-21-5	Lactic acid	0.12-0.03	0.17-0.06	0.7-0.5
24P	79-14-1	Glycolic acid	0.12-0.02	0.13-0.04	0.9-0.6
25P	96-48-0	g-Butyrolactone	0.11-0.04	0.02-0.01	5.0-3.6
26P	110-15-6	Succinic acid	0.09-0.05	0.14-0.11	0.4-0.6
27V	124-40-3	Dimethylamine	0.05-0.03	0.010-0.008	5.1-3.7
28P	65-85-0	Benzoic acid	0.027-0.051	0.028-0.014	0.9-0.6
29P	91-22-5	Quinoline	0.022-0.004	0.002-0.0005	11.0-8.0
30P	57-88-5	Cholesterol	0.019	0.022	0.9
31P	62-53-3	Aniline	0.011	0.00036	30.0
32P	581-49-7	Anatabine	0.010-0.0002	0.020-0.002	0.5-0.1
33P	16543-55-8	Nitrososornicotine	0.009-0.0001	0.003-0.002	3.0-0.5
34P	486-84-0	Harman	0.005-0.0012	0.003-0.0017	1.7-0.7
35V	463-58-1	Carbonyl sulfide	0.005-0.001	0.070-0.018	0.1-0.03
36V	62-75-9	N-Nitrosodimethylamine	0.0040-0.0002	0.00004-0.00001	100.0-20
37P*		NNK	0.0040-0.0001	0.00100-0.00010	4-1
38P	95-53-4	2-Toluidine	0.0030	0.00016	19.0
39P	7440-02-0	Nickel	0.0024-0.00026	0.00008-0.00002	30.0-13
40V	55-18-5	N-Nitrosodiethylamine	0.0010	0.000025	40.0
41V	936-55-2	N-Nitrosopyrrolidine	0.00090-0.00004	0.00003-0.000006	30.0-6
42P	7440-43-9	Cadmium	0.00072	0.00010	7.2
43P	7440-66-6	Zinc	0.00040	0.00006	6.7
44P	56-55-3	Benz[a]anthracene	0.00028-0.00004	0.00007-0.00002	4.0-2
45P	92-67-1	4-Aminobiphenyl	0.00014	0.000005	31.0
46P	50-22-8	Benzo[a]pyrene	0.00014-0.00005	0.00004-0.00002	3.5-2.5
47V	302-01-2	Hydrazine	0.00009	0.00003	3.0
48P	116-54-7	N-Nitrosodiethanolamine	0.00008-0.00002	0.00007-0.00002	1.2-1.0
49P	91-59-8	2-Naphthylamine	0.00005	0.000002	30.0
50P	7440-68-1	Polonium-210	pCi 0.4-0.04	pCi 0.10-0.04	4.0-1

* 3-Pyridyl-3-3-(N-methyl-N-Nitrosoamino)propylketone

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substances are labeled as "P", to mean particulates. Line 3P (total particulates) has a per cigarette range from 76 to 20 mg SSS, 40 to 15 mg MSS and SSS/MSS ratio of 1.9 to 1.3. The major component of particulates is in Line 5P (nicotine) ranging per cigarette from 8.2 to 2.6 mg for SSS and 2.5 to 1.0 per cigarette for MSS. Most of the remaining particulate constituents are in microgram quantities (see line 27V for dimethylamine, 50 to 30 microg); polycyclic aromatic amines and metallic constituents are in nanogram amounts (Lines 41V to 50P). Most vapor constituents have SSS higher than MSS emission; thus, SSS/MSS ratios exceed 1 (see highest ratio of 170 for Line 4V, ammonia). However, it should be noted that relatively higher SSS emissions are not directly inhaled by nonsmokers but diluted with air in the enclosure, adsorbed in room furnishings, and discharged in the ventilating system. The tabulated MSS quantities apply to nonfiltered cigarettes and are considerably reduced by incorporation of filters.

Sidestream Constituents with Workplace Standards

More than half of the 50 selected constituents are useful industrial chemicals and have threshold limit values (Table 3). The 26 constituents are arranged in the order of increasing estimated cigarette equivalents, starting with

- nicotine, then
- acrolein,
- formaldehyde,
- carbon monoxide, and
- ammonia.

Their cigarette equivalents are less than 10, i.e., it takes 8 or less cigarettes to approach the corresponding TLV consumed in an enclosed nonventilated space of 10 cubic meters. For (v) hydrazine, (w) aniline, (x) dimethylamine, (y) acetone, and (z) 2-toluidine, the cigarette equivalents exceed 1,000. The situation seems impossible because the amount of oxygen in a 10 cubic meter enclosure will not support combustion of 1,000 or more cigarettes. The most extreme equivalent of 29,600 cigarettes can be dismissed as ridiculous, because 29,600 cigarettes would almost completely fill 10 cubic meters of enclosed space. Therefore, a consideration of TLVs can not support the allegation that any one or more of the fifty selected constituents in ETS can cause smoking-associated diseases in nonsmokers. There is a margin of safety of 10 or more times TLV for an initial biologic effect to appear, and 100 to 1,000 times for poisoning and death. The target organs include mucosal lining, nervous system, lungs, skin, eyes, kidneys, liver and blood.

Sidestream Constituents Without Workplace Standards

The polycyclic aromatic amines, including benz(a)anthracene and benzo(a)pyrene, are formed during the combustion of organic matter and fuels. In animal experiments, repeated oral, dermal, subcutaneous or intratracheal administrations cause tumors (Table 4). However, inhalations of the same tabulated polycyclic amines or polonium, simulating human exposure to SSS or MSS, do not result in pulmonary tumors. A true inhaled carcinogen causes pulmonary lesions in both experimental animals and exposed humans. For example, bischloromethyl ether (BCME) and chloromethyl methyl ether (CMME) are proven inhaled tumorigens because human neoplasms seen in workers can

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Table 3. Sidestream constituents with workplace standard

Chemical Name	Initial* Maximum		TLV mg/cum	Cigarette equivalent	Line
	lethal	SSS mg/cig			
(a) Nicotine	N/N	8.2	0.5	0.8	5P
(b) Acrolein	M/P	1.5	0.25	1.7	11V
(c) Formaldehyde	M/P**	5.0	1.5	3	8V
(d) Carbon monoxide	B/N	108	55	5	2V
(e) Ammonia	M/P	22	18	8	4V
(f) Nitrogen oxide	M/N	6.0	30	50	7V
(g) Hydroquinone	O/N	0.27	2	74	20P
(h) Acetic acid	M/P	2.9	25	86	9V
(i) Formic acid	M/P	0.78	9	115	15V
(j) Particulates	M/P	76	1,850	138	3P
(k) Pyridine	M/H	0.8	15	188	14V
(l) Carbon dioxide	N/N	440	9,000	204	1V
(m) Nickel	M/P	0.024	0.1	417	39P
(n) Phenol	M/P	0.42	19	452	18P
(o) Toluene	N/B	8.0	375	470	6V
(p) Methyl chloride	M/N	1.98	105	530	10V
(q) Catechol	D/K	0.32	20	617	19V
(r) Benzene	N/B**	10.0	30	625	16V
(s) Methylamine	M/N	0.18	12	672	21V
(t) Cadmium	M/P	0.00072	0.05	700	42P
(u) Hydrogen cyanide	B/N	0.12	11	880	22V
(v) Hydrazine	M/H**	0.00009	0.1	1,040	47V
(w) Aniline	B/B	0.011	8	4,400	31P
(x) Dimethylamine	M/H	0.05	18	6,250	27V
(y) Acetone	M/N	1.25	1,780	14,240	12V
(z) 2-Toluidine	M/B	0.003	9	29,600	38P

* Initial effects/lethal target organs: B = Blood; D = Dermal; H = Hepatic; K = Kidney; M = Mucosal irritation; N = Nervous system; P = Pulmonary.

** Suspected tumorigen

be reproduced by inhalation exposure or experimental animals to BCME or CMME [15]. The suspected tumorigens in SSS and MSS administered by inhalation of tobacco smoke do not induce pulmonary neoplasm in experimental animals.

The last group of SSS constituents without workplace standards have been tested for mutagenicity. The results of mutagen testing have been positive for six constituents, and negative for seven others (Table 5). All of them have not been tested by inhalation route, and available biological activity are derived from oral, parenteral injection, or dermatomucosal application in experimental animals.

Conclusions

The consensus selection by the USPHS, NAS-NRS and IARC of fifty constituents in environmental tobacco smoke has been reviewed in terms of potential health effects. It is

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Table 4. Sidestream constituents without workplace standards: suspected tumorigens based on oral, dermal or intratracheal administration in experimental animals

Chemical name	Maximum SSS mg	Route	Line
4-Aminobiphenyl	0.00014	oral, subcutaneous	45P
Benz(a)anthracene	0.00028	dermal, subcutaneous	44P
Benzo(a)pyrene	0.00014	oral, dermal, tracheal	46P
2-Naphthylamine	0.00005	oral, subcutaneous	49P
NNK*	0.004	Mutagen	37P
N-Nitrosodimethylamine	0.004	oral, dermal	36V
N-Nitrosodiethylamine	0.001	oral, dermal, tracheal	40V
N-Nitrosodiethanolamine	0.00008	oral, subcutaneous	48P
N-Nitrosopyrrolidine	0.0009	oral	41V
N-Nitrosornicotine	0.009	oral, subcutaneous	33V
Polonium - 210	pCi 0.4	tracheal	50P

*3-pyridyl-3-(N-methyl-N-Nitrosamino)propylketone.

Table 5. Sidestream constituents without workplace standards: unsuspected tumorigens; some positive genotoxicity

Chemical name	Maximum SSS mg	Genotoxicity	Line
Anatabine	0.010	Negative	32P
Benzoic acid	0.027	Mutagen	28P
g-Butyrolactone	0.11	Negative	25P
Carbonyl sulfide	0.005	Negative	35V
Cholesterol	0.019	Mutagen	30P
Glycolic acid	0.12	Negative	24P
Harman	0.005	Mutagen	34P
Lactic acid	0.12	Mutagen	23P
3-Methylpyridine	0.47	Negative	17V
Quinoline	0.022	Mutagen	29P
Succinic acid	0.09	Mutagen	26P
3-Vinylpyridine	1.20	Negative	13V
Zinc	0.0004	Negative	43P

this reviewer's opinion that the selected constituents do not cause smoking-associated diseases in nonsmokers. The concentrations in sidestream smoke are so low that respective threshold limit values may be exceeded by igniting less than 10 cigarettes in 10 cubic meter enclosure for nicotine, acrolein, formaldehyde, carbon monoxide and ammonia. However, this is unlikely to occur in public places or dwellings. It will take 55 to 29,600 ignited cigarettes in a 10 cubic meter enclosure to exceed the respective TLV for 21 other constituents. The remaining 24 constituents with no recommended work standards have been tested for tumorigenicity and genotoxicity. Polycyclic aromatic amines do not cause pulmonary tumors when administered by inhalational route in experimental animals. Other constituents have not been investigated by inhalation route but instead, by oral, subcutaneous injection, or dermatomucosal application. Finally, it

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should be noted that with the exception of carbon dioxide, carbon monoxide, particulates, ammonia and nicotine, the 44 other remaining constituents are present in cigarette smoke in microgram and nanogram quantities. Since the total emission is so small and diluted by environmental air, it is unlikely that any one of the fifty selected constituents can be a health hazard to the nonsmokers. Potentiation between two or more constituents has not been proven or disproven.

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